Inflammation-induced depression

Studying the role of proinflammatory cytokines in anhedonia

Floor van Heesch

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Studying the role of proinflammatory cytokines in anhedonia

Inflammatie-geïnduceerde depressie

Onderzoek naar de rol van proinflammatoire cytokines in anhedonie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 15 januari 2014 des middags te 4.15 uur

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Voor mijn ouders

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General introduction

Psychoneuroimmunology and sickness behavior

For a long time immunologists and neuroscientists considered the central nervous system (CNS) to be an immune privileged organ, separated from immune cells by the blood brain barrier (BBB). The awareness of reciprocal pathways between immune, endocrine and CNS started with the observation of Ader and co-workers showing that immunosuppression can be behaviorally conditioned (Ader and Cohen, 1975). Soon thereafter researchers discovered that cytokines play a key role in the bidirectional communication pathway between the immune system and the brain. In this thesis our focus is on the immune-to-brain pathway.

Cytokines, produced by the peripheral immune system, affect the brain via multiple routes (Dantzer et al., 2008; Konsman et al., 2002):

- First of all, cytokines can directly cross the BBB via a saturable transport system (*carrier route*) (***Figure 1.1: b***) (Banks et al., 1991; Gutierrez et al., 1993);
- At circumventricular organs (CVOs), where the capillary bed does not form a strong BBB, cytokines can enter the brain via simple diffusion (*humoral route*) (***Figure 1.1: c***) (Quan et al., 1998);
- Alternatively, cytokines can disrupt the BBB and increase its permeability (*humoral route*) (Ellison et al., 1987);
- Furthermore, cytokines can increase the production of cyclooxygenase-2 (COX-2) in endothelial cells of the BBB leading to synthesis of prostaglandins. In turn, lipophilic prostaglandins can freely cross the BBB to produce their inflammatory effects in the brain (*humoral route*) (***Figure 1.1: d***) (Cao et al., 1996);
- The vagus nerve can be activated by proinflammatory cytokines as well, leading to activation of the nucleus tractus solitaries in the brain stem (*neural route*) (***Figure 1.1: e***) (Konsman et al., 2000).

In this way, cytokines can signal to the brain and produce serious changes in behavior. These behavioral changes of the organism to infection and inflammation, is known as sickness behavior. Characteristics of sickness behavior are malaise, depressed mood, fever, loss of appetite and weight loss, sleeping problems, inability to concentrate and decreased interest in the surroundings (Dantzer et al., 2008; Konsman et al., 2002). These CNS responses to an infection mimic symptoms of major depression (see section: “Major depression and depression due to a general medical condition”) and are considered to promote animal survival. However, in people with severe infections or chronic inflammatory diseases, an active immune system is thought to increase the risk of developing a depression. Indeed, it has been shown that the prevalence of major depression is significantly higher in patients suffering from infections, rheumatoid arthritis, inflammatory bowel disease or psoriasis (Akay et al., 2002; Benros et al., 2013; Hauser et al., 2011; Isik et al., 2007; Loftus et al., 2011).

The hypothesis that enhanced levels of proinflammatory cytokines increase the risk of depression is strengthened by several observations:

- First of all, it has been demonstrated that interferon α (IFN-α) and interleukin 2 (IL-2) therapies for the treatment of hepatitis C or cancer, significantly increase the risk to develop a depression (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987);
- Furthermore, therapies with tumor necrosis factor α (TNF-α) antibodies not only decrease symptoms of inflammatory diseases, but also symptoms of major depression (Krishnan et al., 2007; Tyring et al., 2006; Uguz et al., 2009);
- Accordingly, depressed patients commonly display alterations in their immune system, including increased levels of TNF-α and IL-6. Adjacent to it, although to a lesser extent, IL-1 has also been associated with depression (Dowlati et al., 2010; Howren et al., 2009);
- Moreover, the antidepressant sertraline (a selective serotonin reuptake inhibitor (SSRI)) showed to decrease serum levels of proinflammatory cytokines (TNF-α, IL-2 and IL-12), whereas serum levels of anti-inflammatory cytokines were increased (IL-4 and transforming growth factor-β1 (TGF-β1)) in a subgroup of depressed patients (Sutcigil et al., 2007). A meta-analysis also revealed that SSRIs, while efficacious decreasing depressive symptoms, also reduced serum TNF-α and IL-6 levels (Hannestad et al., 2011a).

Altogether this makes further studying of an etiological relationship between proinflammatory cytokines and a depressed mood worthwhile.

Major depression and depression due to a general medical condition

According to the World Health Organization (WHO), depression affects about 350 million people worldwide and leads to suicide in approximately 1 million people each year. To be diagnosed with major depression, five or more of the following symptoms should be present over a two-week period or longer. The two core symptoms of major depression are 1) a depressed mood and 2) anhedonia and at least one of these two must be present. A depressed mood is defined as feeling sad or empty or tearful, whereas anhedonia is defined as the inability to experience pleasure from normally pleasurable experiences. Other symptoms include 3) significant weight loss or weight gain, 4) insomnia or increased desire to sleep, 5) restlessness or slowed behavior, 6) fatigue or loss of energy, 7) feelings of worthlessness or excessive inappropriate guilt, 8) trouble making decisions or trouble thinking or concentrating and 9) recurrent thoughts of death or suicide attempt (APA, 1994). Psychosocial treatment can be successfully used to treat depression, but especially in severe cases of depression antidepressants such as SSRIs and serotonin-norepinephrine reuptake inhibitors (SNRIs) are prescribed as well. Unfortunately, approximately 30% of

depressed patients do not fully respond to antidepressants (Kulkarni and Dhir, 2009). This might be due to a high variability in risk factors for major depression and therefore high variability in etiology as well. For instance, known risk factors for major depression are being female, being young (25-35), being divorced, being separated, having marital discord, having a family history of depression, being exposed to stressful life events and having an autoimmune disease and/or being exposed to severe infections (Benros et al., 2013; Kendler et al., 1999; Weissman, 1987). Due to this high variability in risk factors it is conceivable that major depression has different etiologies as well. Especially in case of infections, autoimmune diseases and other general medical conditions associated with an active immune system, it is imaginable that proinflammatory cytokines are involved in the etiology of depression.

According to DSM IV, a depressed mood or anhedonia that is the direct physiological consequence of a specific general medical condition must be distinguished from a major depression. Instead, these patients should be diagnosed with "depression due to a general medical condition" (APA, 1994). However, first it must be proven that the depression is directly caused by a specific general medical condition and is not caused by stress associated with the medical condition or side effects of medication, drugs of abuse or toxin exposure. Furthermore, the disturbances in mood should not exclusively occur during the course of a delirium, or acute confusional state (APA, 1994). Since proinflammatory cytokines are upregulated in a substantial amount of general medical conditions, TNF-α antibodies not only alleviate symptoms of inflammatory diseases, but also symptoms of major depression (Krishnan et al., 2007; Tyring et al., 2006; Uguz et al., 2009) and therapies with IFN-α and IL-2 increase the risk of a depression (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987), it is plausible that there is an etiological relationship between proinflammatory cytokines and a depressed mood/anhedonia. However, the exact pathophysiological mechanisms behind proinflammatory cytokine-induced changes in mood are largely unknown. To find an effective treatment for this (sub)group of depressed patients it is important to get a better understanding of the pathobiological mechanisms underlying inflammation-induced depression.

Lipopolysaccharide (LPS)

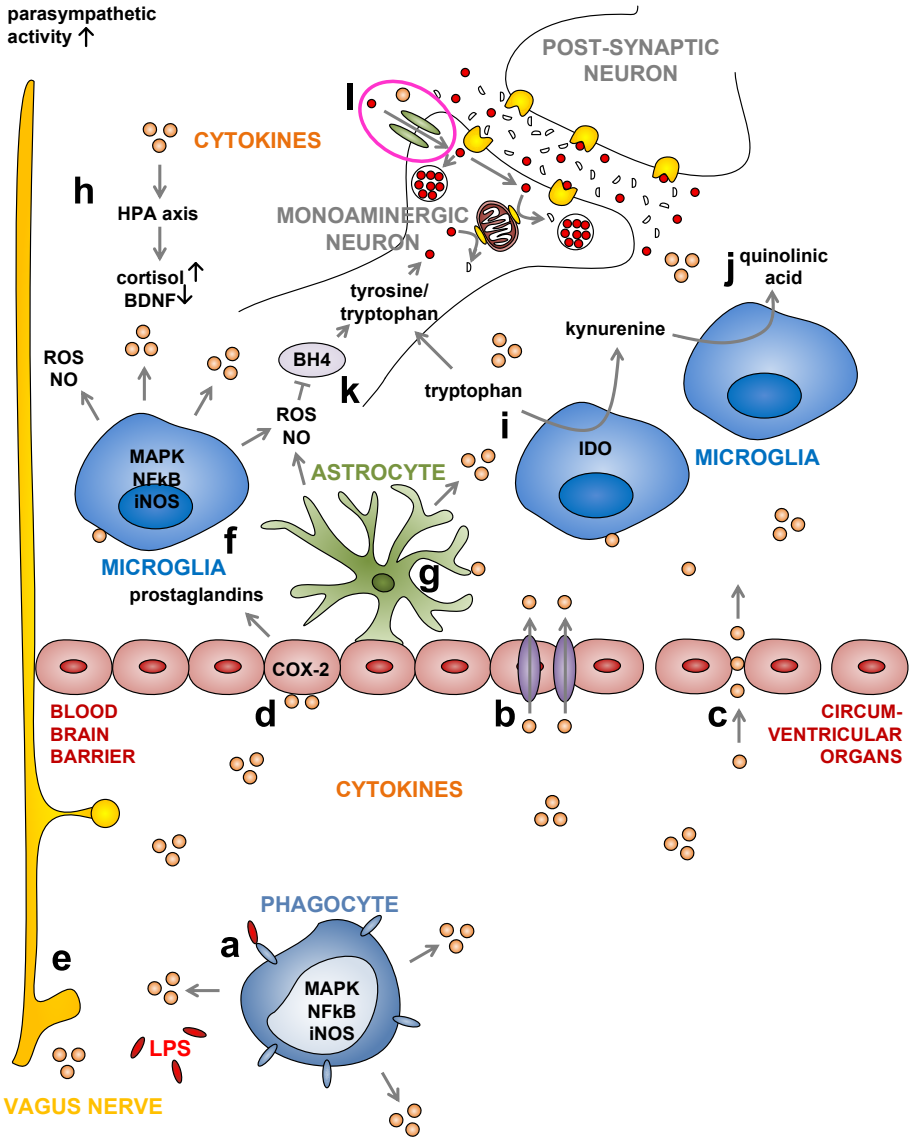
As mentioned before, it has been demonstrated that infections increase the risk of mood disorders dramatically. Individuals with 5 or more hospital contacts with different types of infections even had a higher risk of subsequent mood disorders (Benros et al., 2013). Furthermore, major depression is accompanied by immune responses against gram negative bacteria. This immune response is the result of bacterial translocation from the intestines to the blood, a phenomenon described as leaky gut. In this way, gram negative gut commensals infiltrate in tissues and initiate a systemic immune response. This bacterial

translocation may be a primary trigger factor associated with the onset of major depression or might occur secondary to systemic inflammation in depression and may exacerbate the mental disorder (Maes et al., 2008; Maes et al., 2012). To activate the innate immune system and provoke immune-activated anhedonia in our *in vivo* animal models, *Escherichia Coli* derived lipopolysaccharide (LPS) was injected intraperitoneally. LPS is known to be among the most powerful activators of the innate immune system in mammalian species. This essential and major component of the outer membrane of gram negative bacteria consists of a polysaccharide or oligosaccharide domain and an immunostimulatory lipid A domain that is anchored to the gram negative bacterial membrane. Toll-like receptor 4 (TLR4), that is localized on the surface of phagocytes (monocytes, macrophages and neutrophils) and dendritic cells, is specialized in the recognition of the lipid A domain of LPS. Activation of the TLR4-MD-2 complex by lipid A results in activation of intracellular signaling pathways including mitogen-activated protein kinase (MAPK) signaling cascades and the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling pathway leading to activation of innate immunity. Amongst others proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 are upregulated (***Figure 1.1: a***) (for a detailed review see: Alexander and Rietschel, 2001). These proinflammatory cytokines can initiate a complex network of secondary reactions, for instance the initiation of a bidirectional network between cells of the immune system and the brain leading to sickness behavior, including anhedonia (Dantzer et al., 2008; Konsman et al., 2002). In order to investigate inflammation-induced anhedonia, in most of the experiments conducted in this thesis *Escherichia Coli* derived LPS was used to activate the innate immune system of rats and mice and induce a proinflammatory cytokine response (**Chapter 2**, **4**, **5** and **6**).

Tumor necrosis factor-alpha (TNF-α)

A proinflammatory cytokine that seems to be highly associated with "depression due to a general medical condition" is TNF-α. TNF-α levels are significantly increased in depressed patients (Dowlati et al., 2010) and SSRIs are able to inhibit these levels (Hannestad et al., 2011a; Sutcigil et al., 2007). Furthermore, unlike depressed patients that did not respond to the tricyclic antidepressant amitriptyline, serum TNF-α levels were significantly reduced in depressed patients that responded to amitriptyline (Lanquillon et al., 2000). Moreover, as mentioned before, it has been shown that treatment with TNF-α antibodies in chronic inflammatory diseases not only relieve symptoms of the chronic inflammatory disease itself, but also reduce symptoms of depression in these patients (Krishnan et al., 2007; Tyring et al., 2006; Uguz et al., 2009). Interestingly, TNF-α is the most principle cytokine that mediates acute and chronic inflammation and is highly upregulated after exposure to LPS. Therefore, we investigated whether TNF-α is able to induce comparable changes in mood as LPS. Hence, **Chapter 3** is devoted to this proinflammatory cytokine.

CENTRAL NERVOUS SYSTEM



PERIPHERY

Figure 1.1: A simplified overview: How peripherally (LPS-induced) cytokines signal to the brain and induce changes that are potentially involved in the development of a depressed mood and anhedonia. a) Peripherally injected lipopolysaccharide (LPS) binds to Toll-like receptor 4 and activates intracellular signaling pathways, including mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and inducible nitric oxide synthase (iNOS) leading to activation of innate immunity and production of cytokines. These peripherally produced cytokines (orange dots) can signal to the brain via multiple routes: **b)** via saturable transport systems (*carrier route*); **c)** via simple diffusion at circumventricular organs (CVO) (*humoral route*); **d)** via stimulation of prostaglandin synthesis by endothelial cells of the blood brain barrier (*humoral route*); or **e)** via activation of the vagus nerve (*neural route*). In the central nervous system, proinflammatory cytokines stimulate **f)** microglia and **g)** astrocytes to release and produce cytokines in the brain (*cellular route*). Cytokines induce changes in the brain that are potentially involved in the development of a depressed mood and anhedonia: **h)** the cytokine-induced activated vagus nerve and proinflammatory cytokines activate the hypothalamus-pituitary-adrenal (HPA) axis leading to increased levels of cortisol and decreased levels of brain-derived neurotrophic factor (BDNF); **i)** proinflammatory cytokines stimulate indoleamine 2,3-dioxygenase (IDO) leading to catabolism of tryptophan to kynurenine, possibly impairing serotonin (5-HT) (red dots) synthesis; **j)** kynurenine can be further metabolized into the neurotoxic compound quinolinic acid; **k)** activated microglia and astrocytes release reactive oxygen species (ROS) and nitric oxide (NO) leading to inhibition of tetrahydrobiopterine (BH4), potentially suppressing the production of monoamines (red dots); **l)** furthermore proinflammatory cytokines are known to increase monoamine transporter activity leading to increased metabolism of monoamines (white half dots). In this thesis special attention is given to the role of monoamine transporters in LPS-induced anhedonia (**l**).

Intracranial self-stimulation (ICSS)

To investigate whether peripheral administration of LPS (**Chapter 2**, **4** and **5**) and TNF-α (**Chapter 3**) induce anhedonia in rats and mice we used the intracranial self-stimulation (ICSS) procedure. This technique is able to assess the sensitivity of the reward system directly. In other words, with ICSS it is possible to assess whether a manipulation leads to a decreased ability to experience reward (anhedonia) or an increased ability to experience reward (hedonia) (Carlezon and Chartoff, 2007; Stoker and Markou, 2011). ICSS thresholds, i.e. the lowest electrical intensity at which an animal responds, were obtained according to the discrete-trial current-intensity procedure as described before (Kornetsky and Esposito, 1979; Kenny et al., 2003). To obtain brain stimulation reward, animals turn a wheel in response to a non-contingent stimulus. By doing this, an electrode that is implanted in the lateral hypothalamus of the brain is activated. Electrical stimulation of this brain area leads to activation of the medial forebrain bundle, a group of fibers that carries information to the nucleus accumbens (NAc). The NAc is highly innervated by dopaminergic fibers arising from the ventral tegmental area (VTA) and is considered to be the reward center of the brain playing a role in anhedonia (Nestler and Carlezon, 2006). Indeed, it has been demonstrated that the NAc is smaller and less sensitive for reward in depressed patients (Pizzagalli et al., 2009; Wacker et al., 2009). In line with this, higher

intensities for brain stimulation reward are necessary to make an anhedonic animal respond to the non-contingent stimulus and turn the wheel in the ICSS procedure to self-administer a second electrical stimulation. This is reflected by increased ICSS thresholds implying decreased responding for brain stimulation reward. Conversely, in hedonic animals less electrical stimulation of the lateral hypothalamus is necessary to activate the reward center of the brain and make the animal turn the wheel in response to the non-contingent stimulus. In this case, responding for brain stimulation reward is increased and this is reflected by decreased ICSS thresholds (Stoker and Markou, 2011). Interestingly, baseline ICSS thresholds are constant over time (Stoker and Markou, 2011) and therefore ICSS is a good method to assess the possible anhedonic effects of LPS and TNF-α at several time points after peripheral injection.

Body weight

During each ICSS experiment, body weight was measured as well (**Chapter 2**, **3**, **4** and **5**). This was done in order to demonstrate that activation of the innate immune system by LPS or TNF-α administration not only leads to anhedonia, but also to other symptoms of sickness behavior, such as loss of body weight probably due to loss of appetite.

Monoamines

Monoamines, such as serotonin (5-HT), dopamine (DA) and norepinephrine (NE), are neurotransmitters that transmit signals from a neuron to a target cell. 5-HT is derived from the essential amino acid l-tryptophan, whereas DA and NE are derived from l-tyrosine, a non-essential amino acid. Synthesis of 5-HT, DA and NE predominantly takes place in serotonergic, dopaminergic and norepinephrinergic neurons, respectively (Deutch and Roth, 2008; Eisenhofer et al., 2004). After production, 5-HT, DA and NE are stored in vesicles and/or can function as neurotransmitters. In the latter case the monoamine is released by the presynaptic neuron into the synaptic cleft and can bind to receptors on pre- and postsynaptic neurons. Depending on the receptor that is stimulated the pre- or postsynaptic neuron is stimulated or inhibited (Deutch and Roth, 2008; Eisenhofer et al., 2004). In this way, monoamines are able to modulate neuronal activity and it is known that alterations in monoamine signaling lead to alterations in behavior and mood.

Monoamine transporters

Monoamines play an important modulatory role in neuronal communication. As long as monoamines are present in the synaptic cleft, receptors are stimulated. Monoamine transporters are able to diminish this signal by transporting monoamines back into the

presynaptic neuron. Each monoamine has its own monoamine transporter; 5-HT is taken up by the serotonin transporter (SERT), DA by the dopamine transporter (DAT) and NE by the norepinephrine transporter (NET) (Deutch and Roth, 2008; Eisenhofer et al., 2004). After reuptake, monoamines are stored in storage vesicles for reuse later on, or are broken down in mitochondria by the enzyme monoamine oxidase (MAO). In the latter event, 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) (***Figure 1.2: A***), DA to 3,4-dihydroxy-phenylacetic acid (DOPAC) (***Figure 1.2: B***) and NE to 3,4-dihydroxyphenylglycol (DHPG). Subsequently, 5-HIAA, DOPAC and DHPG diffuse out of the neurons (Deutch and Roth, 2008; Eisenhofer et al., 2004). 5-HIAA is the major metabolite of 5-HT (Deutch and Roth, 2008). DOPAC, on the other hand, is further metabolized by catechol-*O*-methyltransferase (COMT) to homovanillic acid (HVA) in glial cells (Eisenhofer et al., 2004) ***(Figure 1.2: D)***. In brain areas where DAT density is low, e.g. the medial prefrontal cortex (mPFC) (Sesack et al., 1998), reuptake of DA takes place via NET (Gresch et al., 1995; Gu et al., 1994) ***(Figure 1.2: C)***. Alternatively, DA can also be metabolized in glial cells, without the necessity of DAT (or NET). In this case DA is metabolized by COMT to 3-methyoxytyramine (3-MT) followed by oxidation to HVA by MAO (Eisenhofer et al., 2004) ***(Figure 1.2: D)***. After formation, these monoaminergic metabolites, that are biologically inactive, pass the BBB and are excreted in urine. Thus, monoamine transporters play an important role in the regulation of serotonergic, dopaminergic and norepinephrinergic neurotransmission and in the formation of monoaminergic metabolites.

Interestingly, current antidepressants predominantly interfere with monoamine signaling in the brain leading to increased levels of monoamines in the synaptic cleft. For example, 1) SSRIs block the reuptake of 5-HT by SERT, 2) norepinephrine reuptake inhibitors (NRIs) block the reuptake of NE by NET, 3) SNRIs block the reuptake of both 5-HT and NE by SERT and NET, respectively, 4) triple reuptake inhibitors (TRIs) block the reuptake of 5-HT, NE and DA by SERT, NET and DAT, respectively, and 5) monoamine oxidase (MAO) inhibitors lead to decreased metabolism of all three monoamines. This suggests that alterations in central monoamine signaling are highly involved in major depression. Therefore, in this thesis, we want to explore whether peripheral activation of the innate immune system by LPS (**Chapter 2**, **4** and **6**) or systemic administration of the proinflammatory cytokine TNF-α (**Chapter 3**) leads to alterations in central monoamine signaling that is possibly related to the development of anhedonia.

Proinflammatory cytokines and monoamine signaling in the brain

As mentioned previously, peripherally produced cytokines signal to the brain via several routes. Consequently, microglia (***Figure 1.1: f***) and astrocytes (***Figure 1.1: g***) (Chung and Benveniste, 1990; Lieberman et al., 1989), but also neurons (Schobitz et al., 1992) are stimulated to release and produce proinflammatory cytokines in the brain (*cellular route*).

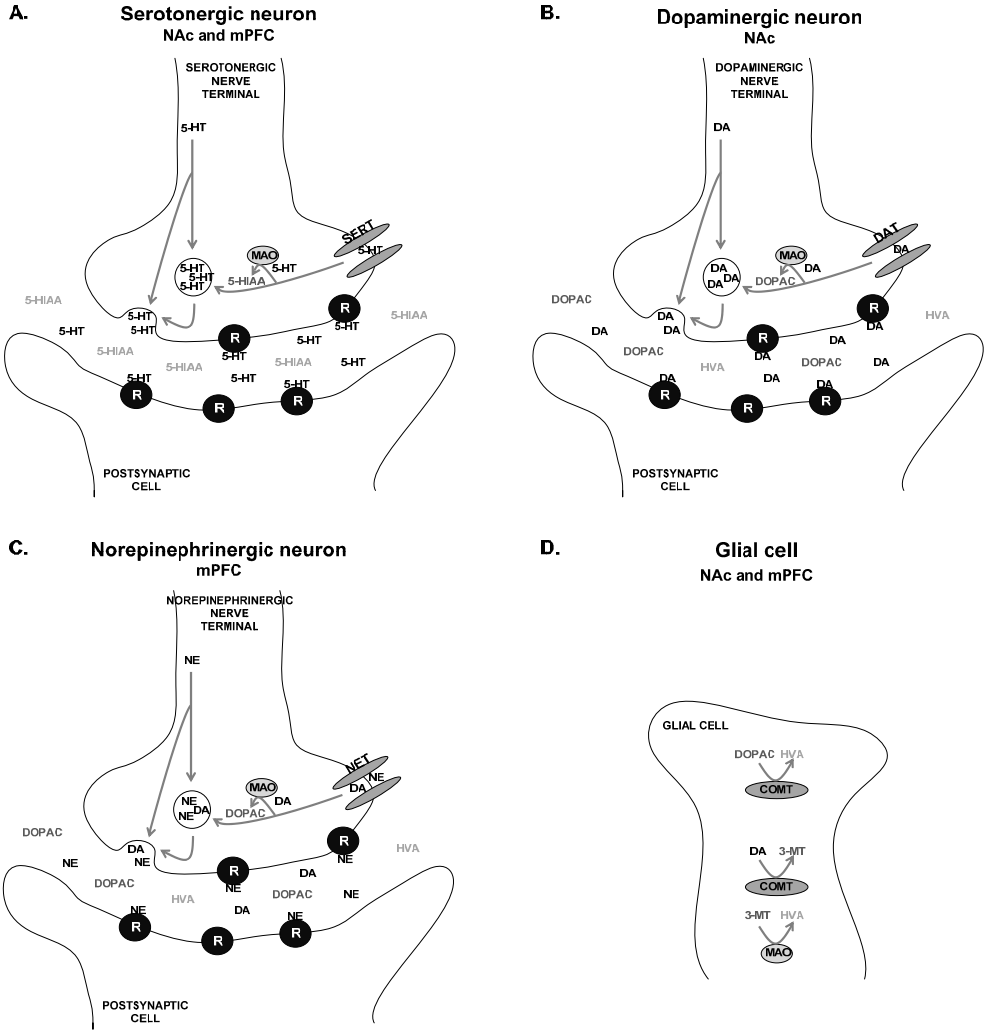


Figure 1.2: A simplified overview of the formation of serotonergic and dopaminergic metabolites in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). (A) After reuptake by the SERT, 5-HT is metabolized to 5-HIAA by MAO. 5-HIAA, in turn, is released into the synaptic cleft. This happens in NAc as well as in the mPFC. **(B)** In the NAc DA is taken up by DAT. After reuptake DA is metabolized to DOPAC by MAO that is released into the synaptic cleft (see (D) for the proceeding steps of dopamine metabolism). **(C)** Since no DAT is expressed in the mPFC, DA is taken up by NET. After reuptake DA is metabolized in norepinephrinergic neurons. **(D)** In the NAc as well as in the mPFC, glial cells are important for the formation of HVA. DOPAC that is released by dopaminergic (and norepinephrinergic) neurons is metabolized to HVA by COMT. Furthermore, glial cells can metabolize DA without the necessity of DAT (or NET). In this case, COMT metabolizes DA to 3-MT. 3-MT, in turn, is metabolized to HVA. After formation, HVA is released by the glial cell.

Microglia and astrocytes, i.e. the "housekeepers" of the brain, are responsible for the control of pathogen infiltration and clearance of toxic and apoptotic cell debris. After exposure to LPS these cells start to secrete proinflammatory cytokines (Jensen et al., 2013; Yang et al., 2010). It has been hypothesized that these cytokines can induce changes in the brain via several pathways that might be involved in the development of a depressed mood and anhedonia (***Figure 1.1***). Most of these pathways directly or indirectly lead to changes in monoamine signaling:

- First of all, it is known that activation of the vagus nerve by peripheral proinflammatory cytokines, as well as locally produced proinflammatory cytokines in the brain activate the hypothalamus-pituitary-adrenal (HPA) axis leading to increased levels of cortisol (***Figure 1.1: h***) (Wieczorek et al., 2005; Wieczorek and Dunn, 2006). Stress is highly associated with major depression (Kendler et al., 1999) and leads to alterations in monoamine signaling as shown for instance by a blunted accumbal DA response, and to some extent, a blunted accumbal 5-HT response to cocaine following chronic social defeat stress (Mangiavacchi et al., 2001; Shimamoto et al., 2011). Furthermore proinflammatory cytokines, LPS, stress and HPA-axis activity are known to decrease levels of brain-derived neurotrophic factor (BDNF) (***Figure 1.1: h***) (Guan and Fang, 2006; Kenis et al., 2011) and this has been associated with major depression (Kunugi et al., 2010; Shimizu et al., 2003);
- It is known that proinflammatory cytokines in the brain stimulate microglia and to a lesser extend astrocytes to produce the enzyme indoleamine 2,3-dioxygenase (IDO) (Kwidzinski et al., 2005). IDO is responsible for the catabolism of tryptophan to kynurenine, leading to tryptophan deficiency that likely impairs 5-HT synthesis and 5-HT concentrations in the brain, possibly contributing to the development of anhedonia (***Figure 1.1: i***) (Maes et al., 2011). Furthermore, kynurenine, known to induce depressive-like behavior in the forced swim test (O'Connor et al., 2009), is metabolized into the neuroactive metabolites quinolinic acid and kynurenic acid. Quinolinic acid, produced by microglia and acting as an NMDA agonist, does have neurotoxic effects (***Figure 1.1: j***), whereas kynurenic acid, produced by astrocytes acting as an NMDA antagonist, has neuroprotective effects. In line with this, Raison and co-workers found an association between IFN-α-induced synthesis of central kynurenine and quinolinic acid in cerebrospinal fluid (CSF) and the development of depressive symptoms, whereas there was no such an association between kynurenic acid and the development of depressive symptoms (Raison et al., 2010);
- Another route to induce structural changes in the brain is via the production of nitric oxide (NO) and reactive oxygen species (ROS) by activated microglia and astrocytes in response to proinflammatory cytokines and LPS (Zielasek and Hartung, 1996). Intramuscular IFN-α administration to rats has been shown to

stimulate NO that was associated with decreased concentrations of tetrahydrobiopterin (BH_4) and DA in the amygdala and raphe areas. Additional treatment with an inhibitor of NO synthase reversed IFN-α inhibitory effects on brain concentrations of both BH_4 and DA (Kitagami et al., 2003). Since BH_4 is an important cofactor for tryptophan hydroxylase and tyrosine hydroxylase, i.e. the rate limiting enzymes for the synthesis of 5-HT, DA and NE, respectively, monoaminergic neurotransmission is expected to be affected via this route as well (***Figure 1.1: k***);

- Another important target of proinflammatory cytokines are monoamine transporters. An increasing amount of evidence points to a role for increased SERT activity in response to proinflammatory cytokines (***Figure 1.1: l***) (Mossner et al., 1998; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010). Furthermore, SSRIs have been shown to relieve depression and reduce serum levels of proinflammatory cytokines (Hannestad et al., 2011a; Sutcigil et al., 2007). Less is known about the effect of proinflammatory cytokines on DAT and NET activity. Since increased activity of monoamine transporters will lead to weakening of the monoaminergic signal and increased availability of monoamines for degradation of 5-HT and DA to 5-HIAA and DOPAC, respectively, by MAO, it is to be expected that cytokines affect monoaminergic neurotransmission via this route as well. In this thesis we will focus on the involvement of monoamine transporters in LPS-induced anhedonia.

Microdialysis

Microdialysis studies were performed to investigate how extracellular monoamine (metabolite) levels are affected by peripheral activation of the innate immune system and by the cytokine TNF-α (**Chapter 2**, **3**, **4** and **6**). To perform microdialysis in the brain, a small microdialysis probe with a semipermeable membrane is placed in the brain area of interest, in our case the NAc and medial prefrontal cortex (mPFC). Both brain areas were chosen because of their involvement in major depression. The NAc plays a pivotal role in reward and anhedonia (Nestler and Carlezon, 2006) whereas the mPFC is important in overall cognitive functioning and suppression of negative affect (Robbins and Arnsten, 2009). One day after probe placement the animal is connected to the microdialysis system. During microdialysis experiments, artificial cerebrospinal fluid (CSF) is continuously pumped into the brain via the probe. Small molecules, e.g. 5-HT, 5-HIAA, DA, DOPAC and HVA, can cross the semipermeable membrane via passive diffusion. Based on this principle, dialysates containing monoamines and monoamine metabolites are collected at 30 minute intervals for analysis. During a challenge that interferes with monoamine signaling in the brain, in our case an intraperitoneal injection with LPS or TNF-α, changes in monoamines and monoamine metabolites are observed. The possibility to measure

changes in monoamine and monoamine metabolite levels in the brain over time, before and during (a) manipulation(s) in a living, awake animal is an advantage of microdialysis (Westerink, 1995). Furthermore, by measuring the metabolites of 5-HT and DA as well, we get a better insight in the role of monoamine transporters in LPS- and TNF-α-induced anhedonia (see section: "Monoamine transporters").

Aim and outline of the thesis

The aim of this thesis was to get a better insight in monoaminergic neurotransmission during inflammation-induced anhedonia. Our focus was on the role of monoamine transporters, and consequently the production of metabolites in this process. In **Chapter 2** we validated our model for LPS-induced anhedonia in rats and investigated how monoaminergic signaling is affected in the NAc and mPFC. In addition, we have studied the effect of a single cytokine, TNF-α, on brain stimulation reward and brain monoaminergic signaling in a mouse model in **Chapter 3**. In **Chapter 4** we first show that LPS induces anhedonia in mice as well, after which we investigate the effect of the triple reuptake inhibitor DOV 216,303 (inhibits SERT, DAT and NET) on LPS-induced alterations in monoamine metabolite levels. This was done in order to investigate the role of monoamine transporters in LPS-induced changes in monoamine metabolite levels. To dig deeper into the role of SERT in LPS-induced anhedonia and alterations in monoamine signaling in the brain, the effect of LPS on sensitivity of the reward system in serotonin transporter knockout rats is investigated in **Chapter 5**, whereas the effects of LPS on serum and forebrain cytokine levels and accumbal and prefrontal 5-HT and 5-HIAA levels in this animal model are presented in **Chapter 6**. Finally, in **Chapter 7** all our findings are summarized and discussed.

2

Lipopolysaccharide lowers brain stimulation reward and increases extracellular monoamine metabolite levels: Anhedonia or sickness behavior?

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Abstract

Lipopolysaccharide (LPS) has been shown to reduce brain stimulation reward in rats, suggesting that LPS induces anhedonia, i.e. the inability to experience pleasure, which is a core symptom of major depressive disorder. Since peripheral LPS is known to induce reductions in motor activity as well, it is important to check whether inflammation-induced disturbed motor activity affects the outcome of these ICSS studies. Furthermore, given that monoaminergic neurotransmission affects behavior and mood, we planned to investigate how peripheral LPS affects monoamine signaling in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), brain areas involved in the pathophysiology of major depressive disorder. Therefore, the effects of an intraperitoneal LPS injection on extracellular monoamine metabolite concentrations was investigated by *in vivo* microdialysis in the NAc and mPFC of male Wistar rats. Furthermore, effects of LPS on ICSS thresholds, distance moved in the open field and body weight were measured. LPS significantly reduced brain stimulation reward (up to 72 h after LPS), and reduced distance moved in the open field and body weight. Since LPS did not increase ICSS response latencies, we conclude that LPS-induced reductions in brain stimulation reward reflect a state of anhedonia. We conclude that LPS-induced anhedonia and other symptoms of sickness behavior peak 4 h after LPS but that LPS-induced anhedonia persists longer. Furthermore, LPS significantly increased formation of monoamine metabolites in the NAc and mPFC. The latter is in agreement with earlier findings that proinflammatory cytokines enhance serotonin transporter activity, and alter dopaminergic neurotransmission.

Introduction

Since the discovery of reciprocal pathways between the immune system and the brain (Ader, 1980), more and more evidence points to a causal role of proinflammatory cytokines in the development of major depression (Dantzer et al., 2008; Konsman et al., 2002; Yirmiya et al., 2000). Patients with chronic inflammations, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis, have an increased risk to become depressed (Akay et al., 2002; Hauser et al., 2011; Isik et al., 2007; Loftus et al., 2011). Interestingly, therapies with tumor necrosis factor alpha (TNF-α) antibodies have shown to reduce depressive symptoms in these patients (Krishnan et al., 2007; Tyring et al., 2006; Uguz et al., 2009). Moreover, it has been demonstrated that the use of interleukin 2 (IL-2) or interferon alpha (IFN-α) for the treatment of hepatitis C or cancer, increased the risk to develop major depression (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987). Overall this suggests that an active immune system plays an important role in the development of major depression.

In many animal studies lipopolysaccharide (LPS), an essential component of the outer membrane of gram negative bacteria, is used as a potent activator of the immune system leading to the rapid release of proinflammatory cytokines. LPS-induced systemic inflammation is known to induce behavioral changes including fever, fatigue, sleepiness, pain, reductions in motor activity, decreased appetite and loss of body weight, social withdrawal, feelings of worthlessness and depressed mood (Dantzer et al., 2008; Konsman et al., 2002). These behavioral changes, known as sickness behavior, have much in common with symptoms of major depression as described in DSM IV (APA, 1994).

A core symptom of major depression is anhedonia, i.e. the inability to experience pleasure. Anhedonia can be quantified by measuring decreased sensitivity for brain stimulation reward using an intracranial self-stimulation (ICSS) procedure (Carlezon and Chartoff, 2007). LPS has been shown to reduce brain stimulation reward 1 h to 24 h after peripheral injection (Barr et al., 2003; Borowski et al., 1998; van Heesch et al., 2013a). Previously, it has been hypothesized by Dantzer and co-workers that depressive-like behavior gradually emerges on the background of LPS-induced sickness behavior and that LPS-induced depression-like behavior peaks 24 h after peripheral LPS exposure, whereas other symptoms of sickness behavior are already decreased at this time point (Dantzer et al., 2008). In the present study we investigated the time course in which the LPS-induced reductions in brain stimulation reward in the rat occur and whether the peak is indeed observed 24 h post-treatment.

It is well known that alterations in monoamine signaling affect mood, given the fact that most antidepressants act on monoaminergic neurotransmission. Two brain areas in particular are known to be affected in major depression; the nucleus accumbens (NAc) and

medial prefrontal cortex (mPFC). The NAc is highly involved in reward and anhedonia (Nestler and Carlezon, 2006), whereas the mPFC is known to suppress negative affect (Robbins and Arnsten, 2009). Since peripheral LPS has been shown repeatedly to decrease brain stimulation reward in rats (Barr et al., 2003; Borowski et al., 1998; van Heesch et al., 2013a), we hypothesize that peripheral LPS also affects neurochemistry in these two brain structures.

To summarize, we will first show the time course of LPS-induced reductions in brain stimulation reward in an ICSS paradigm in rats. To demonstrate that the dose of LPS used in our studies induces neurovegetative and somatic symptoms of sickness behavior as well, we also measured alterations in body weight and distance moved in the open field at several time points after exposure to LPS. According to these results the interpretation of ICSS data as a measure for LPS-induced anhedonia is discussed. Finally we present results of a microdialysis study in the NAc and mPFC in order to show how LPS affects monoaminergic neurotransmission that possibly underlies anhedonia.

Methods

Animals

Seventy-six male Wistar rats (Harlan, Horst, the Netherlands), weighing between 290 – 350 grams at the time of surgery, were socially housed, four per cage. Animals were placed on a 12 h light/dark cycle with lights on at 6:00 am and off at 6:00 pm. Food and water were available *ad libitum*. Rats had at least one week to acclimate to their new environment. All experiments were carried out in accordance with the governmental guidelines and approved by the Ethical Committee for Animal Research of Utrecht University, The Netherlands.

Drugs

Escherichia Coli derived lipopolysaccharide (LPS) (Sigma, 0127:B8) was dissolved in saline and prepared freshly on test days from a stock solution (0.5 mg/ml dissolved in saline). Rats received 250 µg/kg LPS intraperitoneally (i.p.) in a volume of 1 ml/kg. Control rats were injected with saline i.p. in a volume of 1 ml/kg.

Brain stimulation reward

ICSS surgery

Twenty rats were anesthetized by inhalation of isoflurane gas (2%), mixed with oxygen and placed in a stereotaxic instrument. Lidocaine hydrochloride (2%) + adrenaline (0.001%) were applied in the incision as local anesthetic. Twisted bipolar electrodes (cut to 11 mm) were implanted in the lateral hypothalamus (anteroposterior – 0.5 mm; mediolateral +/– 1.7 mm (under a 0^{o} angle) from bregma; dorsoventral -8.3 mm from dura (Pellegrino et al., 1979)). During surgeries, the incisor bar was adjusted to 5 mm above the interaural line (Pellegrino et al., 1979). Electrodes were anchored with four screws and dental acrylic on the skull. All animals received the non-steroidal anti-inflammatory drug (NSAID) Rimadyl for pain relief (5 mg/kg, subcutaneously, twice a day, four injections in total). Throughout the entire experiment, rats were housed four per cage. Before start of ICSS training animals were given one week to recover from surgery.

ICSS training

One week after surgery rats were trained daily in the ICSS paradigm according to the discrete-trial current-intensity procedure as described before by Kornetsky and Esposito (1979) and Kenny et al. (2003). Each session consisted of four alternating series of descending and ascending current intensities starting with a descending series. A set of five trials was presented for each current intensity and the intensity was altered in 5 µA steps between blocks of trials. The initial current intensity was set approximately 40 µA above baseline thresholds. One-quarter turn of the wheel manipulandum within 7.5 sec after the presentation of a non-contingent electrical stimulation (positive response) resulted in another electrical stimulus. The time to respond to the non-contingent electrical stimulus was recorded (response latency). After a positive or negative response (no turn of the wheel within 7.5 sec after the non-contingent stimulus) the inter-trial interval started with an average duration of 10 sec (7.5-12.5 sec range) followed by another trial with the delivery of a non-contingent stimulus. The onset of the next trial was delayed in case animals responded during the inter-trial interval. Animals that were unable to learn the ICSS program during the training sessions were excluded from the ICSS experiment (n = 3).

Set-up of the ICSS experiments

After the establishment of steady ICSS thresholds (6 consecutive days during which ICSS thresholds varied by <10%), rats received an i.p. injection of either saline (n = 9) or LPS (n = 8) and were tested in the ICSS paradigm 1 h, 4 h, 24 h, 48 h, 72 h, 144 h and 168 h after

injection. During each measurement ICSS thresholds and response latencies were recorded. ICSS thresholds and ICSS response latencies measured during the six days before saline or LPS injection were averaged and used to calculate percentage change from baseline values at each time point tested after the injections. Increased ICSS thresholds compared to baseline measurements implies that brain stimulation reward is decreased. After the last measurement, animals were decapitated.

Neurovegetative and somatic symptoms of sickness behavior

Distance moved in the open field

Locomotor activity of 24 rats was assessed in the open field at 4 different time points; at baseline and 4 h, 72 h and 144 h after exposure to saline (n = 12) or LPS (n = 12). During each test, rats were placed in a squared arena (92 x 92 x 40 cm [l x w x h]), with an open top, dark walls and a dark floor. Position of the animal and total distance moved (m) during a 10 min trial was automatically tracked (TSE system, Germany) .

Body weight

Body weight of 16 rats was measured daily around noon. Body weight of rats was measured during three days before and 24 h, 48 h, 72 h, 144 h and 168 h after the saline (n = 8) or LPS (n = 8) injection. Body weight measured during the three days before saline or LPS injection was averaged and used to calculate body weight at baseline and assess delta body weight values at different time points after the saline or LPS injection. After the last body weight measurement, animals were decapitated.

Monoamine signaling in the NAc and mPFC

Microdialysis surgery

Sixteen rats were anesthetized by inhalation of a mixture of isoflurane gas (2%) and oxygen and placed in a stereotaxic instrument. Cuprofane microdialysis probes, MAB 4.6.2 CU and MAB 4.7.3 CU (Microbiotech) were implanted simultaneously in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), respectively. The coordinates were anterioposterior +1.6 mm; mediolateral +1.8 mm (under a 0^{o} angle) from bregma; dorsoventral –8.2 mm from skull surface for the NAc and anterioposterior +3.2 mm; mediolateral +1.0 mm (under a 0^{o} angle) from bregma; dorsoventral –4.0 mm from skull

surface for the mPFC (G. Paxinos, 2001). Probes were anchored with nonacrylic dental cement on the scull. After implantation of the microdialysis probes, rats were housed individually and placed in the microdialysis room until the end of the experiment.

Set-up of the microdialysis experiment

The microdialysis experiment was performed in conscious freely moving rats, one day after implantation of the microdialysis probes. A pump (KdScientific Pump 220 series, USA) perfused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant flow rate of 0.02 ml/h. During microdialysis, the flow rate was set at 0.09 ml/h. At 8:00 am rats were connected to a dual channel swivel (type 375/D/22QM) which allowed them to move freely. Three h after connection 30-minute samples were manually collected in vials containing 15 µl of 0.1 M acetic acid frozen at –80 ^{o}C until analysis with HPLC. From 11:00 am until 1:00 pm four baseline samples were collected. Subsequently, the animals were injected i.p. with saline (n = 8) or LPS (n = 8) and samples were collected for an additional 4 h. During the whole experiment, 12 samples were collected in total. At the end of the microdialysis experiment all animals were sacrificed immediately. The brains were dissected and stored in formaldehyde to verify probe localization later on.

HPLC

Microdialysis samples were stored at –80 °C until analysis. Neurotransmitters, serotonin (5-HT) and dopamine (DA) and their metabolites, 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), respectively, were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, The Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 x 50 mm, 3 µm particle size) in combination with detector cell 1, separated and detected 5-HT and DA. Column 2 (NeuroSep 115 C18 1 x 150 mm, 3 µm particle size) in combination with detector cell 2, separated and detected the metabolites. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 18.5 % Methanol and 400 mg/L OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5 % methanol and 700 mg/L OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. The neurotransmitters were detected electrochemically using µVT-03 flow cells (Antec) with glassy carbon working electrodes. Potential settings were for 5-HT and DA +0.30 V versus Ag/AgCl and for metabolites +0.59 V versus Ag/AgCl. The columns and detector cells

were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec). The limit of detection was 0.05 nM (S/N ratio 3:1).

Probe placement

Dissected brains were quickly stored in formaldehyde until verification of probe localization. The brains were placed in 30% sucrose solution and frozen two days later. Probe placements were verified on 60 µm cresyl violet stained sections obtained on a cryostat (Leica CM3050).

Statistical analysis

To verify that groups were equal before exposure to saline or LPS, baseline ICSS thresholds, baseline response latencies, baseline total distance moved in the open field, baseline body weight and mean baseline levels of 5-HT, 5-HIAA, DA and HVA in the NAc as well as in the mPFC were analyzed with use of independent *t*-tests. Furthermore, microdialysis baseline measurements were analyzed by repeated measures analysis of variance (repeated measures ANOVA) with time (4 levels: –120 min, –90 min, –60 min and –30 min) as within subject factor and treatment (saline or LPS) as between subject factor.

ICSS current-threshold data and response latency data are expressed as a percentage of the baseline data, distance moved in the open field data are expressed as absolute values, whereas body weight data are expressed as the difference from baseline data (delta body weight). NAc and mPFC microdialysis data were analyzed separately and are expressed as a percentage of the baseline data. ICSS thresholds, response latencies, distance moved in the open field, delta body weight and microdialysis data were analyzed by repeated measures ANOVA with time as within subject factor and treatment (saline or LPS) as between subject factor. The different levels for time were: 8 levels: 0 h, 1 h, 4 h, 24 h, 48 h, 72 h, 144 h and 168 h (ICSS thresholds and response latencies); 4 levels: 0 h, 4 h, 72 h and 144 h (open field); 6 levels: 0 h, 24 h, 48 h, 72 h, 144 h and 168 h (delta body weight); or 9 levels: 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min and 240 min (microdialysis). In case of a significant time x treatment interaction effects of LPS on individual time points were analyzed with post hoc *t*-tests. When the assumption of sphericity was violated, reported results were corrected by the Greenhouse-Geisser procedure. All data were analyzed using the SPSS 20 software statistical package.

Results

Brain stimulation reward

ICSS thresholds

Mean absolute baseline ICSS thresholds (± S.E.M.) were 114.1 µA (± 8.3) and 113.7 µA (± 18.5) in saline and LPS-treated rats, respectively, and did not differ significantly between treatment groups. After exposure to LPS, repeated measures ANOVA revealed a significant time x treatment interaction ($F(3.2,48.6) = 6.5$, $p = 0.001$, $\varepsilon = 0.463$) and a significant effect of treatment ($F(1,15) = 24.7$, $p < 0.001$) (***Figure 2.1: A***). Post hoc *t*-tests per time point showed that ICSS thresholds were significantly increased (at least $p < 0.05$) 1 h, 4 h, 24 h, 48 h (trend: $p = 0.055$) and 72 h after exposure to LPS.

ICSS response latencies

Response latency is defined as the duration between the delivery of the non-contingent stimulus and the animal's response (turn of the wheel). Mean absolute baseline response latencies (±S.E.M.) were 2.9 s (± 0.2) and 2.6 s (± 0.2) in saline and LPS-treated rats, respectively, and did not differ between groups. Although there was no significant time x treatment interaction, there was a trend towards an effect of treatment ($F(1,15) = 4.1$, $p = 0.060$), suggesting that response latencies were reduced in LPS-treated animals (***Figure 2.1: B***).

Neurovegetative and somatic symptoms of sickness behavior

Distance moved in the open field

Before injections, mean total distance moved during a 10 min trial in the open field did not differ between groups and was (± S.E.M.) 74.5 m (± 9.1) in the saline group and 78.1 m (± 8.9) in the LPS group. Repeated measures ANOVA revelaed a significant time x treatment interaction ($F(3,66) = 2.8$, $p = 0.044$) without a significant effect of treatment. Post hoc *t*-tests per time point revealed that total distance moved was significantly decreased in LPS treated rats compared to saline treated rats at time point 4 h after injection ($p = 0.017$) (***Figure 2.2: A***). This reduction in distance moved was not observed 72 h and 144 h after exposure to LPS.

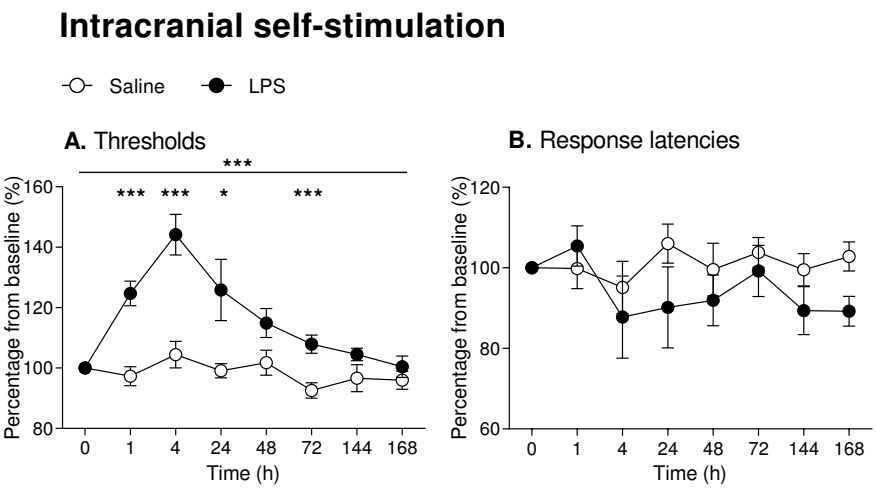

Figure 2.1: The effect of i.p. injected LPS on intracranial self-stimulation (ICSS) tresholds (**A**) and response latencies in rats (**B**). ICSS thresholds and response latencies were measured after exposure to saline (n = 9) or LPS (n = 8). Percentage change from baseline ICSS thresholds and percentage change from baseline ICSS respons latencies are presented as mean ± standard error of the mean; statistically significant results are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to saline-treated animals.

Body weight

In one of the animals loss of body weight was more than 15% of total body weight two days after exposure to LPS. Therefore this animal had to be taken out of the experiment earlier and was excluded from data analysis. As a consequence, the saline group consisted of 8 animals, whereas the LPS group consisted of 7 animals. Before treatment, mean absolute baseline body weight (± S.E.M.) was 577.0 g (± 24.8) in the saline group and 582.6 g (± 23.7) in the LPS group and did not differ between groups. After LPS injection, body weight decreased significantly (***Figure 2.2: B***). There was a significant time x treatment interaction ($F(1.5,20.1) = 23.4$, $p < 0.001$, $\varepsilon = 0.309$) and a significant effect of treatment ($F(1,13) = 37.5$, $p < 0.001$). Post hoc *t*-tests per time point revealed that body weight of LPS treated animals was significantly lower compared to saline treated rats at each time point measured after the injections (at least $p < 0.05$).

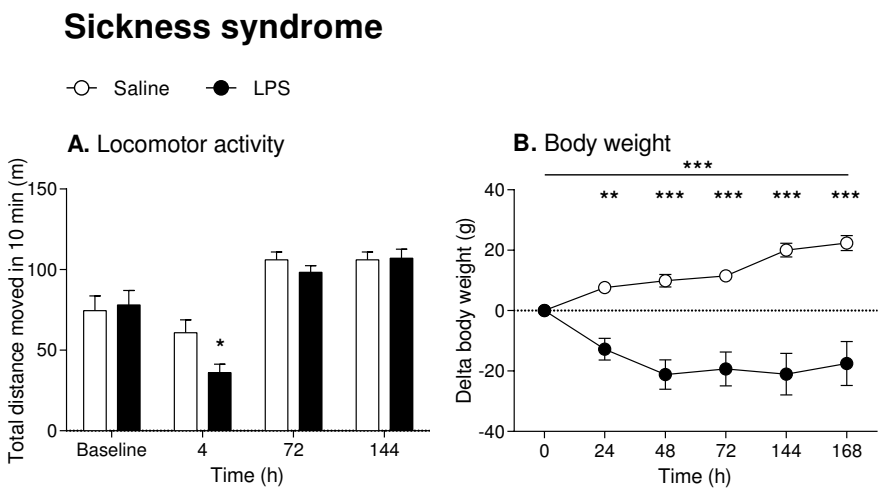

Figure 2.2: The effect of i.p. injected LPS on neurovegetative and somatic symptoms of sickness behavior. Time spent locomoting in the open field (**A**) and delta body weight (**B**) were measured at several time points after exposure to saline (open field: n = 12, body weight: n =8) or LPS (open field: n = 12, body weight: n = 7). Both parameters are presented as mean ± standard error of the mean; statistically significant results are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to saline-treated animals.

Monoamine signaling in the NAc and mPFC

Histology

NAc: One animal was not able to participate in the microdialysis experiment due to an obstructed probe. Furthermore, two animals were excluded from data analysis due to wrong probe placement. Therefore the saline and LPS group consisted of 7 and 6 rats, respectively.

mPFC: In total 3 rats were excluded from data analysis due to missing baseline values. Therefore the saline group consisted of 5 rats, whereas 8 rats were included in the LPS group.

	NAc		mPFC	
	Saline	**LPS**	**Saline**	**LPS**
5-HT (nM)	0.13 ± 0.02	0.15 ± 0.01	0.13 ± 0.03	0.11 ± 0.02
5-HIAA (nM)	199.48 ± 9.86	200.98 ± 22.02	115.72 ± 5.82	117.70 ± 9.69
DA (nM)	2.57 ± 0.82	2.1 ± 0.39	0.39 ± 0.08	0.28 ± 0.05
HVA (nM)	351.79 ± 32.24	383.14 ± 36.29	14.17 ± 1.59	13.92 ± 1.76

Table 2.1: Baseline monoamine and monoamine metabolite levels. Absolute mean baseline 5-HT, 5-HIAA, DA and HVA levels (± standard error of the mean), thus before exposure to saline or LPS, in the NAc and mPFC are presented. Independent *t*-tests revealed no differences in absolute mean baseline monoamine and monoamine metabolite levels between saline and LPS treated mice in both the NAc and mPFC microdialysis study.

Baseline monoamine and monoamine metabolite levels in the NAc and mPFC

In both the NAc and mPFC, absolute mean baseline values of 5-HT, 5-HIAA, DA and HVA did not differ between groups (***Table 2.1***). Furthermore, repeated measures ANOVA for the first four baseline measurements revealed that 5-HT, 5-HIAA, DA and HVA did not differ between the 2 groups in both brain areas. Thus, before exposure to saline or LPS both groups were comparable.

The effect of peripheral LPS on monoamine and monoamine metabolite levels in the NAc

Repeated measures ANOVA revealed neither a significant time x treatment interaction nor an effect of treatment for 5-HT and DA in NAc (***Figure 2.3: A*** and ***Figure 2.3: C***, respectively). However, for 5-HIAA and HVA, the metabolites of 5HT and DA, respectively, there was a significant time x treatment interaction (5-HIAA: $F(3.2,34.9) = 8.5$, $p < 0.001$, $\varepsilon = 0.397$ and HVA: $F(2.3,25.4) = 5.4$, $p = 0.009$, $\varepsilon = 0.289$) and a significant effect of treatment (5-HIAA: $F(1,11) = 17.1$, $p = 0.002$ and HVA: $F(1,11) = 4.9$, $p = 0.048$) (***Figure 2.3: B*** and ***Figure 2.3: D***, respectively). Post hoc *t*-tests per time point showed that LPS significantly increased 5-HIAA at time point 150 min and HVA levels 180 min after exposure to LPS. At the end of the study, i.e. 240 min after exposure to LPS, 5-HIAA and HVA levels were still significantly increased when compared to control rats.

The effect of peripheral LPS on monoamine and monoamine metabolite levels in the mPFC

For 5-HT, repeated measures ANOVA revealed neither a significant time x treatment interaction nor a signficant effect of treatment (***Figure 2.4: A***). Although there was no

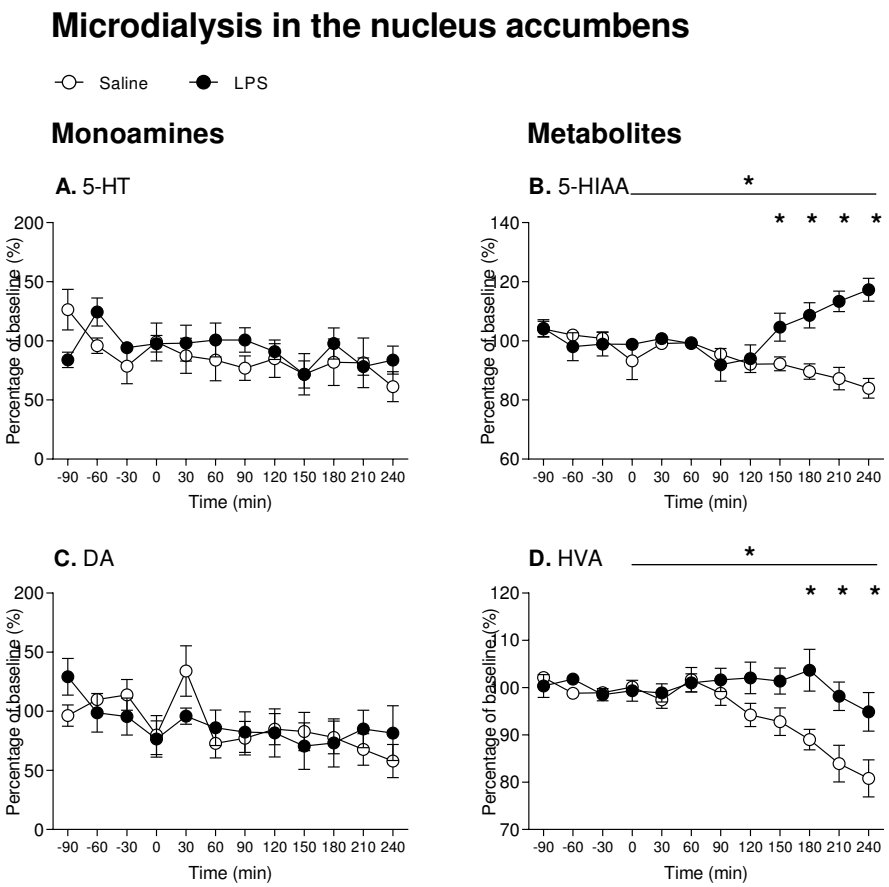

Figure 2.3: The effect of i.p. injected LPS on extracellular 5-HT (**A**), 5-HIAA (**B**), DA (**C**) and HVA (**D**) levels in the NAc was measured under basal condition and after exposure to saline (n = 7) or LPS (n = 6) (time point 0 min). For each monoamine and monoamine metabolite the percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated rats at a single time point are indicated as * $p < 0.05$.

significant time x treatment interaction for DA in the mPFC as well, there was a significant effect of treatment ($F(1,11) = 7.8$, $p = 0.018$) (***Figure 2.4: C***). In correspondance with the observations in the NAc, there was a significant time x treatment interaction for both 5-HIAA and HVA (5-HIAA: $F(2.6,28.4) = 7.0$, $p = 0.002$, $\varepsilon = 0.322$ and HVA: $F(3.3,36.1) = 5.4$, $p = 0.003$, $\varepsilon = 0.411$) (***Figure 2.4: B*** and ***Figure 2.4: D***, respectively). Furthermore there was

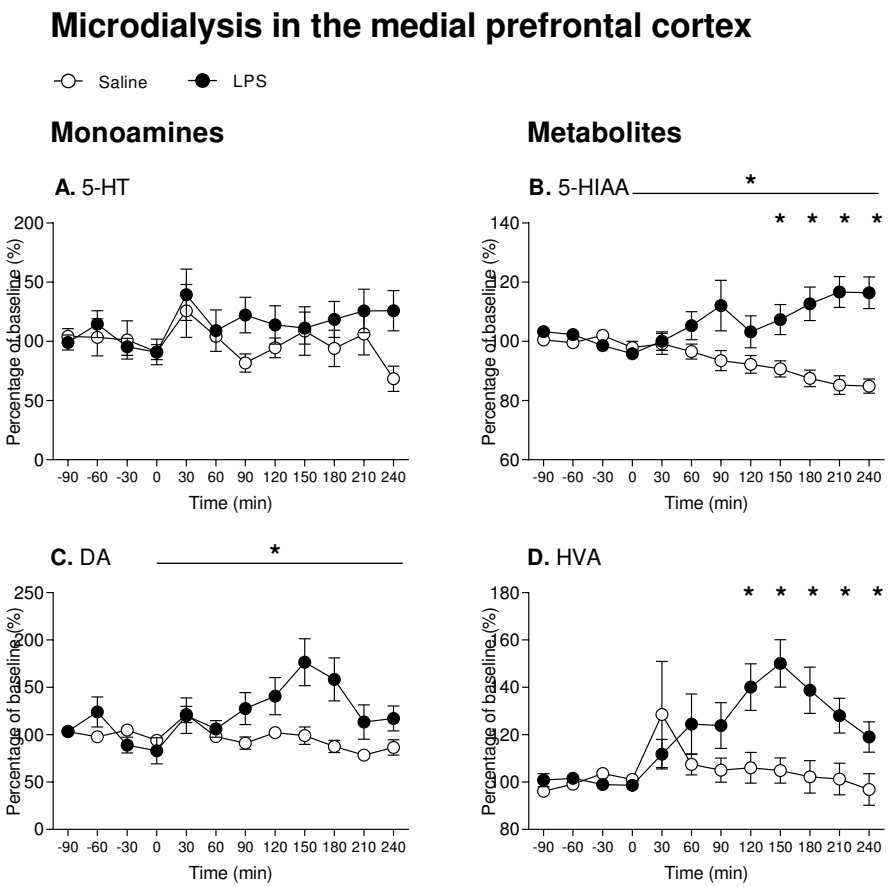

Figure 2.4: The effect of i.p. injected LPS on extracellular 5-HT (**A**), 5-HIAA (**B**), DA (**C**) and HVA (**D**) levels in the mPFC was measured under basal condition and after exposure to saline (n =5) or LPS (n = 8) (time point 0 min). For each monoamine and monoamine metabolite the percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated rats at a single time point are indicated as * $p < 0.05$.

a significant effect of treatment for 5-HIAA and a trend towards an effect of treatment for HVA (5-HIAA: $F(1,11) = 7.7$, $p = 0.018$ and HVA: $F(1,11) = 3.5$, $p = 0.087$). Post hoc *t*-tests per time point showed that 5-HIAA and HVA levels were significantly increased 150 min and 120 min after exposure to LPS, respectively. Both metabolites remained significantly elevated till 240 min after LPS injection.

Discussion

As expected, LPS reduced responding for rewarding brain stimulation. Although LPS decreased locomotor activity and body weight as well, ICSS response latencies tended to decrease in LPS-treated rats, indicating that LPS-induced reductions in brain stimulation reward reflect a state of anhedonia rather than impaired motor functioning. Furthermore, we showed that peripheral LPS significantly increased extracellular levels of serotonin's metabolite 5-HIAA and dopamine's metabolite HVA, without affecting 5-HT in the NAc and mPFC. DA levels in the mPFC increased significantly after LPS treatment.

LPS decreases responding for brain stimulation reward in rats

Previously, it has been demonstrated that brain stimulation reward was decreased 1 h to 24 h after peripheral injection of LPS to rats (Barr et al., 2003; Borowski et al., 1998; van Heesch et al., 2013a). Also in the present study we showed that LPS-induced reductions in brain stimulation reward were present 1 and 24 h after LPS. These reductions persisted significant at 72 h. Highest ICSS thresholds were found 4 h after LPS. Since most cytokines peak between 1 h and 6 h after LPS (Biesmans et al., 2013) and both peripherally injected IL-2 (Anisman et al., 1996; Anisman et al., 1998) and peripherally injected TNF-α (van Heesch et al., 2013b) are able to decrease responding for brain stimulation reward within this time frame, it is likely that LPS-induced cytokines are responsible for the observed decreases in brain stimulation reward in the present study.

LPS-induced reductions in brain stimulation reward reflect anhedonia

LPS is well known to rapidly reduce locomotor activity (Dunn and Swiergiel, 2005) that returns to baseline levels within 24 h (O'Connor et al., 2009). Also in the present study distance moved in the open field was significantly decreased 4 h, but not 72 h and 144 h after exposure to LPS. Being aware of this fact, we should take into account that reductions in motor activity could have affected the outcome of the ICSS study by interfering with performance in the test. However, response latencies revealed that LPS-treated animals compared to saline-treated animals did not respond slower to the non-contingent stimulus. In fact, LPS-treated rats tended to respond faster. This shows that reduced motor performance is not responsible for the observed decreases in responding for rewarding brain stimulation.

Besides reductions in motor activity, LPS produced a significant decrease in body weight. This was anticipated, since LPS and proinflammatory cytokines are known to decrease appetite and food intake, consequently reducing body weight (Langhans and Hrupka, 1999;

Langhans, 2007). Previously, it has been shown in mice that sucrose preference was decreased 6 h, 24 h and 48 h after exposure to an acute or chronic peripheral LPS challenge (Frenois et al., 2007; Kubera et al., 2013). In these and many other studies decreased sucrose preference is interpreted as a measure of anhedonia. Keeping in mind that LPS reduces appetite and food intake, the outcome of these sucrose preference studies might be biased. The current and previous ICSS studies, however, are probably not influenced by LPS-induced loss of appetite, because it is known that rats prefer self-stimulation of the lateral hypothalamus above access to food (Spies, 1965). Furthermore, no food intake is involved in the ICSS procedure.

Since loss of appetite and reductions in motor activity are not likely to affect responding in the present ICSS experiment and since reward and pleasure are highly interconnected, we are convinced that the observed LPS-induced reductions in brain stimulation reward represent anhedonia, i.e. the inability to experience pleasure. In addition, LPS and infections have been shown to produce depressive symptoms in humans as well (Benros et al., 2013; Yirmiya et al., 2000), further suggesting a role for an active immune system in depression.

Previously it has been hypothesized that LPS-induced depressive-like behavior gradually emerges during LPS-induced sickness behavior and reaches its peak 24 h after LPS once other symptoms of sickness behavior are already diminishing (Dantzer et al., 2008). In the present study, however, we showed that most significant LPS-induced reductions in brain stimulation reward occur during the peak of sickness behavior, i.e. 4 h after LPS. Furthermore, we found that reductions in brain stimulation reward were still present 24 h and 72 h after exposure to LPS, suggesting that LPS-induced depressive-like behavior persists longer compared to other symptoms of sickness behavior, such as reductions in locomotor activity. The latter is (in contradiction to the first) in concurrence with the hypothesis of Dantzer and co-workers (Dantzer et al., 2008). Biesmans and co-workers, on the other hand, demonstrated with use of the sucrose preference test that it is not possible to separate sickness behavior from anhedonia in mice (Biesmans et al., 2013). We think, however, that responding for rewarding brain stimulation is a more reliable measurement for inflammation-induced anhedonia.

LPS-induced anhedonia is associated with increased monoamine metabolite levels in the NAc and mPFC

Contrary to our expectations, extracellular levels of 5-HT and DA did not decrease in the NAc and mPFC of rats in response to an LPS dose that does induce anhedonia. In the mPFC, DA levels even increased significantly. This is in line with other microdialysis studies in the NAc and mPFC of rodents showing that extracellular monoamine levels are not affected or even increase in response to a peripheral immune challenge with LPS, IL-1β

or TNF-α (Borowski et al., 1998; Dunn, 2006; Merali et al., 1997; van Heesch et al., 2013b; van Heesch et al., submitted). Furthermore, in a meta-analysis study it has been demonstrated that depletions of 5-HT and DA/NE only lead to depression in subjects with a family history of major depressive disorder or drug-free subjects with major depression in remission, but not in healthy subjects (Ruhe et al., 2007). Thus, more research is needed to understand the complex relationship between monoaminergic neurotransmission and (inflammation-induced) depression.

Although no decreases in monoamine levels were found, we found increased levels of 5-HIAA and HVA, i.e. the metabolites of 5-HT and DA, respectively. Increased levels of accumbal and prefrontal 5-HIAA and HVA have been reported previously after a peripheral injection with LPS (rat and mouse), IL-1β (rat) and TNF-α (mouse) (Borowski et al., 1998; Merali et al., 1997; van Heesch et al., 2013b; van Heesch et al, submitted). Most 5-HIAA is formed after deamination of 5-HT by monoamine oxidase (MAO) in serotonergic neurons via three different routes: 1) A part of newly synthesized 5-HT is metabolized in the neuroplasm before it has been taken up by storage vesicles; 2) Another poule of 5-HIAA could be formed from 5-HT leaking from storage vesicles into the neuroplasm; However, 3) it appears that most 5-HIAA is formed after reuptake of released 5-HT by very efficient serotonin transporters (SERT) (Stenfors and Ross, 2004). So, increased extracellular levels of 5-HIAA in the NAc and mPFC could represent increased SERT activity in both brain areas. Indeed, it has been demonstrated that peripheral LPS, IL-1β and TNF-α increase SERT activity *in vitro* and *in vivo* (Mossner et al., 1998; Ramamoorthy et al., 1995; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010) and there are strong indications that inflammation-induced SERT activity is associated with increased p38 MAPK activity (Baganz and Blakely, 2013; Zhu et al., 2004; Zhu et al., 2010). Furthermore, there are indications that this increased SERT activity is associated with depression-like behavior and anhedonia. First of all, it has been shown that LPS-induced reductions in brain stimulation reward and LPS-induced immobility in the tail suspension test are absent in SERT knockout rats and SERT knockout mice, respectively (van Heesch et al., 2013a; Zhu et al., 2010). Interestingly, SERT knockout rats showed no significant increase in extracellular 5-HIAA levels in the NAc and mPFC after peripheral LPS as well (van Heesch et al., submitted). In addition it has been shown that a 5-day pretreatment with citalopram (selective serotonin reuptake inhibitor) led to a decrease in LPS-induced fatigue and anhedonia in healthy subjects (Hannestad et al., 2011b). Altogether, this suggests that increased SERT activity is involved in LPS-induced anhedonia and this could explain the LPS-induced increases in extracellular 5-HIAA levels in the NAc and mPFC.

In concurrence with 5-HIAA, increased HVA could suggest increased dopamine transporter (DAT) activity as well. However, in contrast to 5-HIAA, HVA can also be produced in glial cells without the involvement of DAT (Eisenhofer et al., 2004). Nevertheless, recently we have shown that LPS-induced increases in accumbal and prefrontal HVA were

abolished once mice were pretreated with the triple reuptake inhibitor DOV 216,303, suggesting that increased DAT and norepinephrine transporter (NET) activity are the cause of increased HVA levels in the NAc and mPFC, respectively (van Heesch et al., submitted). Furthermore, there are indications for increased DAT activity, possibly via activation of p40 and p42 MAPK signaling pathways (Moron et al., 2003; Nakajima et al., 2004). However, it has also been demonstrated that 7-day pretreatment with bupropion (DAT and NET inhibitor) was not able to decrease LPS-induced fatigue and anhedonia in healthy subjects (DellaGioia et al., 2013). This suggests that regulation of DAT and NET activity are less involved in LPS-induced anhedonia compared to SERT activity.

Conclusion

We showed that LPS-induced peripheral activation of the innate immune system rapidly (after 1 h) reduced brain stimulation reward that lasted for 72 h in rats. We argue that these reductions in brain stimulation reward reflect a state of anhedonia. Furthermore, we hypothesize that anhedonia and other symptoms of sickness behavior, e.g. reductions in motor activity and body weight, peak 4 h after exposure to LPS and that LPS-induced reductions in responding for rewarding brain stimulation persists longer (72 h). The LPS-induced increase in monoamine metabolites (5-HIAA and HVA) suggest that SERT and possibly DAT activity are increased. However, we appoint that further molecular studies are needed to investigate the contribution of monoamine transporters and other mechanisms (such as enzyme activity) in inflammation-induced anhedonia.

3

Systemic tumor necrosis factor-alpha decreases brain stimulation reward and increases metabolites of serotonin and dopamine in the nucleus accumbens of mice

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Abstract

Many patients with chronic inflammatory disorders have an abnormal high prevalence of major depression accompanied by elevated levels of tumor necrosis factor-α (TNF-α). We hypothesize that systemic TNF-α increases brain monoamine metabolism, which might induce anhedonia (i.e., a core symptom of major depression). The effect of an intraperitoneal TNF-α injection on extracellular monoamine and metabolite concentrations was investigated by *in vivo* microdialysis in the nucleus accumbens (NAc) of C57BL/6 mice. In another group, the effects of TNF-α on body weight and intracranial self-stimulation (ICSS) thresholds were measured. TNF-α reduced body weight and increased ICSS thresholds, suggesting a state of anhedonia. TNF-α did not affect serotonin levels, but increased its metabolite 5-HIAA in the NAc. Remarkably, TNF-α also increased the dopamine metabolite HVA, without affecting dopamine levels itself. These data concur with earlier findings that proinflammatory cytokines enhance serotonin transporter activity, and possibly also dopamine transporter activity in the brain. However, more research is needed to understand the precise molecular mechanisms by which TNF-α increases transporter activity and anhedonia.

Introduction

Proinflammatory cytokines seem to play a causal role in the pathophysiology of depression. Indeed, patients suffering from chronic inflammatory disorders, having high levels of tumor necrosis factor-α (TNF-α), suffer more from major depression (MD). Biological therapies (e.g. TNF-α antibodies) revolutionized the treatment of inflammatory diseases (Aaltonen et al., 2012; Menter et al., 2010; van Dullemen et al., 1995) and there is evidence that TNF-α antibodies reduce symptoms of depression (Tyring et al., 2006; Uguz et al., 2009). Furthermore, selective serotonin reuptake inhibitors (SSRIs), i.e. the first-line treatment for MD, decrease TNF-α levels (Hannestad et al., 2011a). This suggests that TNF-α itself can cause depression-like symptoms.

The nucleus accumbens (NAc) is part of the mesolimbic dopaminergic reward circuitry playing an important role in anhedonia (Chaudhury et al., 2013; Nestler and Carlezon, 2006; Tye et al., 2013), i.e. the inability to experience pleasure which is a core symptom of MD. Enhanced dopaminergic activity in the NAc has been shown to facilitate self-stimulation in the intracranial self-stimulation (ICSS) paradigm (Olds, 1990). In concordance, IL-2-induced anhedonia, reflected in increased ICSS thresholds, was associated with decreased extracellular dopamine (DA) levels in the NAc (Anisman et al., 1996). However, systemic lipopolysaccharide (LPS), increasing proinflammatory cytokines, including IL-2 and TNF-α, increases both ICSS thresholds and DA levels in the NAc (Borowski et al., 1998). Thus, proinflammatory cytokines induce anhedonia, but how DA levels in the NAc are affected remains unclear. Since TNF-α antibodies and SSRIs decrease serum TNF-α levels and relieve symptoms of MD, the question remains how TNF-α affects accumbal DA and 5-HT levels and how mood, especially anhedonia, is affected. Therefore, in the present study, the effect of TNF-α on monoaminergic neurotransmission was measured by in vivo microdialysis in the NAc and the effect of TNF-α on mood was studied by ICSS.

Methods

Forty male C57BL/6J mice (Charles River, Maastricht) 9 to 10 weeks old were socially housed (8-10/cage) on a 12 h light/dark cycle (lights on from 6:00 am to 6:00 pm). Food and water were available *ad libitum*. Experiments were approved by the Ethical Committee for Animal Research of Utrecht University.

For ICSS, 26 mice were anesthetized by inhalation of isoflurane gas (2%), mixed with oxygen. Lidocaine hydrochloride (2%) and adrenaline (0.001%) were applied to the incision. Twisted bipolar electrodes (Bilaney, cut to 6 mm) were implanted in the lateral hypothalamus with coordinates anteroposterior –0.5 mm; mediolateral +/– 1.3 mm (under a 0^o angle) from bregma; dorsoventral –5.0 mm from skull surface. All animals received Rimadyl for pain relief (5 mg/kg, subcutaneously, twice daily, 4 injections in total). One week after surgery, animals were trained twice daily in the ICSS paradigm using a discrete-trial current-intensity procedure (Johnson et al., 2008). Mice unable to learn the ICSS program during training were excluded. After establishment of steady ICSS thresholds (3 consecutive days during which ICSS thresholds varied by <10%), mice received an i.p. injection of either saline or TNF-α and were tested in the ICSS paradigm 1h, 4h, 24h, 48h, 72h and 96h after injection. During each measurement ICSS thresholds and response latencies were recorded. Body weight of these mice was measured daily around noon. ICSS thresholds, response latencies and body weight of the three baseline measurements were used to calculate percentage change from baseline values at each time point tested after the injections. After the last measurement, animals were sacrificed.

Cuprofane microdialysis probes (Microbiotech, MAB 4.6.1 CU, 1 mm membrane length) were implanted in the nucleus accumbens (NAc) of 14 C57BL/6J mice. The coordinates of the NAc were anteroposterior +1.5 mm; mediolateral +0.8 mm (under an 0^o angle) from bregma; dorsoventral –5.0 mm from skull surface. During microdialysis (as described earlier (Caldarone et al., 2010)), the flow rate was set at 0.07 ml/h. At 8:00 am mice were connected to a channel swivel, allowing them to move freely. Three hours later, 30-min baseline samples were manually collected in vials containing 11.7 µl of 0.1 M acetic acid and frozen at –70 ^{o}C until HPLC-analysis. After 8 baseline samples, mice were injected i.p. with vehicle or TNF-α and another 4 samples were collected. After the experiment brains were dissected and stored in formaldehyde to verify probe localization. Dopamine (DA), serotonin (5-HT) and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), respectively, were analyzed simultaneously by HPLC as described earlier (Prins et al., 2011b) Two days before brain slicing, brains were placed in a 30% sucrose solution. Probe placements were verified on 60 µm cresyl violet stained sections. Animals were discarded if the microdialysis probe was outside the NAc (one animal in both groups).

Escherichia Coli derived murine TNF-α (Immuno Tools, Specific Activity 2 x10^7 IU/mg) was dissolved in 0.9% saline and prepared freshly on test days from a stock solution (100 µg/ml dissolved in miliQ). 50 µg/kg TNF-α dissolved in 0.9% saline was administered i.p. Control animals received injections of 0.9% saline (10 ml/kg).

Baseline body weight, ICSS thresholds, ICSS response latencies and mean NAc baseline monoamine (metabolite) levels were analyzed using independent *t*-tests to verify similarity between the two groups at start of the experiment. Body weight data is expressed as the

difference from baseline (delta body weight), whereas ICSS current-threshold, response latency and microdialysis data are expressed as percentage of baseline. All parameters were analyzed by repeated measures analysis of variance (repeated measures ANOVA) with time (body weight: 6 levels: 0 h, 1 h, 24 h, 48 h, 72 h and 96 h; ICSS thresholds and response latencies: 7 levels: 0 h, 1 h, 4 h, 24 h, 48 h, 72 h and 96 h; microdialysis: 5 levels: 0 min, 30 min, 60 min, 90 min and 120 min) as within subject factor and treatment (saline and TNF-α) as between subject factor. In case of a significant time x treatment interaction, treatment effects on individual time points were analyzed with post hoc *t*-tests with treatment (saline or TNF-α) as grouping variable. When appropriate, reported results were corrected using the Greenhouse-Geisser procedure. All data were analyzed using the SPSS 16.0 software statistical package.

Results

At baseline, body weight, ICSS thresholds, ICSS response latencies and monoamine (metabolite) levels did not differ between groups (data not shown). Body weight of TNF-α-treated mice decreased significantly after injection (***Figure 3.1***). Repeated measures ANOVA revealed a significant time x treatment interaction ($F(2.8,59.8) = 11.9$, $p < 0.001$, $\varepsilon = 0.569$) and significant effect of treatment ($F(1,21) = 23.2$, $p < 0.001$). ICSS thresholds

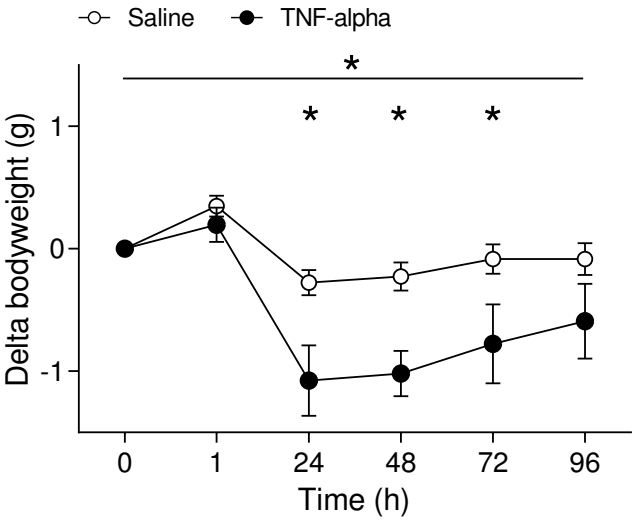

Figure 3.1: The effect of TNF-α (n = 7) versus saline (n = 16) administration on body weight. The figure shows mean delta body weight values at several time points after exposure to TNF-α or saline. * Significant difference between saline and TNF-α treated mice ($p < 0.05$).

increased by TNF-α. Repeated measures ANOVA revealed a significant time x treatment interaction ($F(3.0,63.6) = 4.4$, p = 0.007, $\varepsilon = 0.505$) and significant treatment effect ($F(1, 21) = 4.6$, $p = 0.043$) (***Figure 3.2***). Response latencies, on the other hand, were not affected by TNF-α (data not shown). For 5-HIAA, repeated measures ANOVA revealed a significant time x treatment interaction ($F(4,40) = 4.1$, $p = 0.007$) and significant treatment effect ($F(1,10) = 5.3$, $p = 0.044$) (***Figure 3.3: B***). For HVA, a significant time x treatment interaction ($F(1.9,18.5) = 3.7$, p=0.048, ε = 0.463) was found, but only a trend towards a treatment effect (***Figure 3.3: E***). For DA, a trend towards a time x treatment interaction and treatment effect was observed (***Figure 3.3: C***). Also for DOPAC, a trend towards a time x treatment interaction was found, though there was no treatment effect (***Figure 3.3: D***). For 5-HT, repeated measures ANOVA did neither reveal a time x treatment interaction nor a treatment effect (***Figure 3.3: A***).

Discussion

The finding that TNF-α significantly elevates extracellular 5-HIAA levels without affecting extracellular 5-HT levels in the NAc concurs with earlier reports demonstrating that IL-1

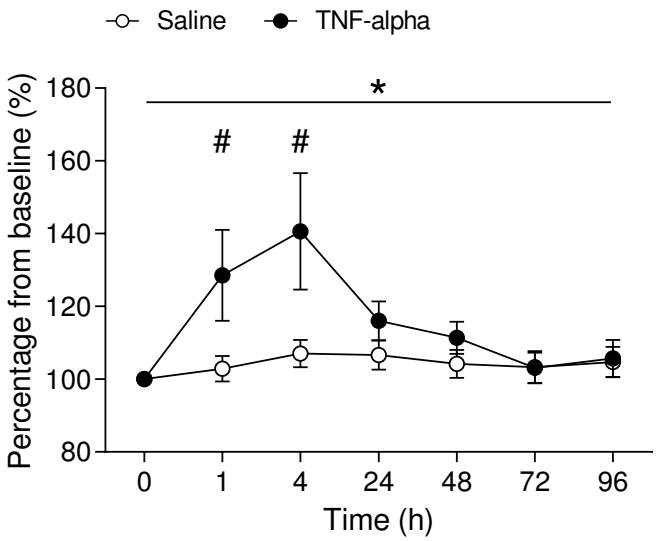

Figure 3.2: The effect of TNF-α (n = 7) versus saline (n = 16) on ICSS thresholds. The figure shows mean percentage change from baseline ICSS thresholds at several time points after exposure to TNF-α or saline. * Significant difference between saline and TNF-α treated mice ($p < 0.05$). # Indicates a trend ($p < 0.1$).

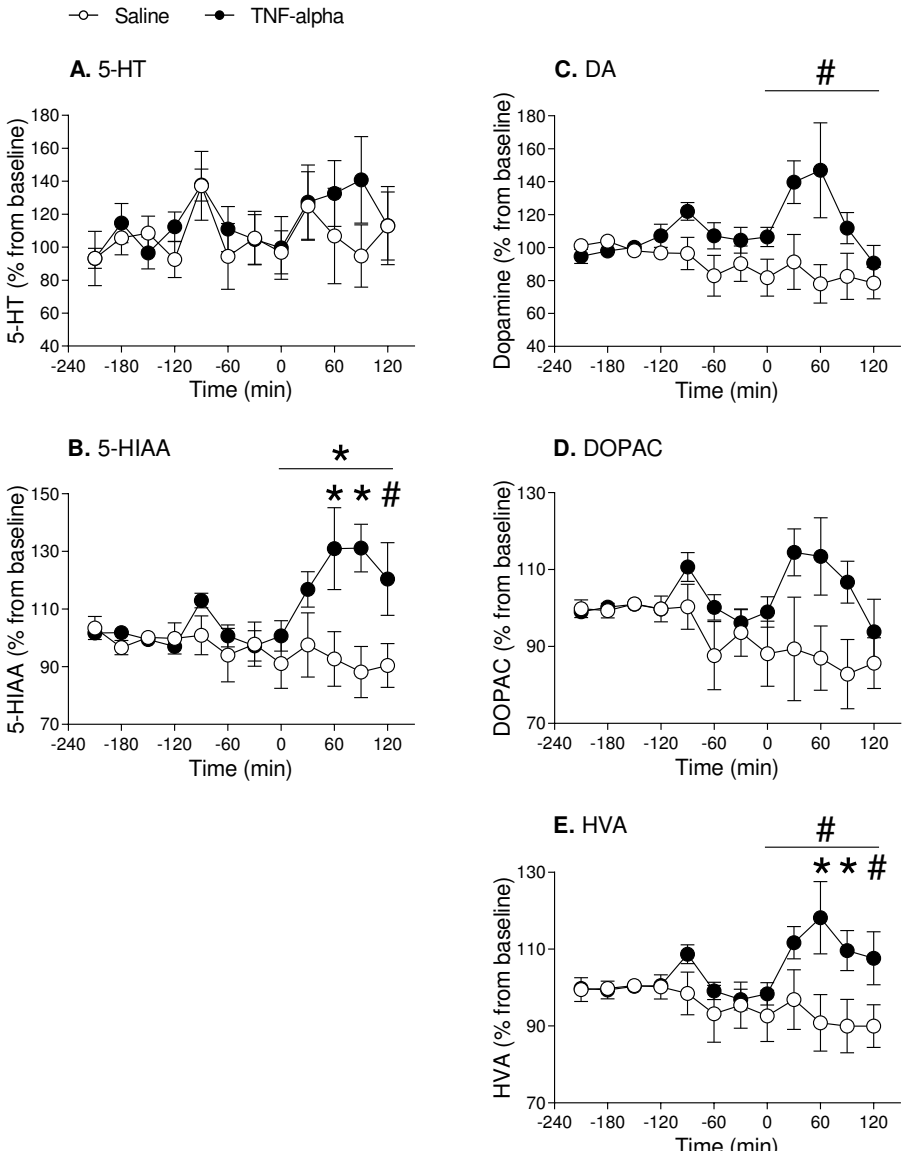

Figure 3.3: The effect of TNF-α (n = 6) versus saline (n = 6) administration on monoamine (metabolite) levels in the NAc. The figure shows mean percentage change from baseline 5-HT (**A**), 5-HIAA (**B**), DA (**C**), DOPAC (**D**) and HVA (**E**) values. * Significant difference between saline and TNF-α treated mice ($p < 0.05$). # Indicates a trend ($p < 0.1$).

and LPS increase accumbal 5-HIAA levels (Borowski et al., 1998; Merali et al., 1997). In the present study, TNF-α might have enhanced serotonin transporter (SERT) function, leading to enhanced 5-HIAA levels in the nucleus accumbens. This is supported by previous *in vitro* and *in vivo* studies showing that TNF-α leads to increased SERT activity, possibly via activation of the p38 MAPK signaling pathway (Baganz and Blakely, 2013; Mossner et al., 1998; Zhu et al., 2006). TNF-α increases SERT function within 20 min by reducing K_m and increasing V_{max} of 5-HT transport (Zhu et al., 2006). In concordance, we showed a rapid increase of 5-HIAA reaching significance within one hour after TNF-α. Interestingly, the antidepressants fluoxetine (SSRI) and imipramine (tricyclic), both blocking SERT, prevent a centrally TNF-α-induced increase in immobility in the forced swimming and tail suspension tests (Kaster et al., 2012), suggesting that increased SERT function is necessary for cytokine-induced depressive-like behavior.

We showed that HVA levels in the NAc were significantly increased 1 h and 1.5 h after TNF-α, without significantly changing DA and DOPAC levels. These results are comparable with the finding that 1.5 h after peripheral administration of IL-1β, extracellular HVA levels are increased in the NAc, without affecting extracellular DA and DOPAC concentrations (Merali et al., 1997). Increased extracellular HVA levels could suggest that DA transport is increased. However, we should be careful in interpreting this finding as increased DAT activity, since HVA, in contrast to DOPAC, can also be produced in glial cells without the necessity of DAT. In other experiments, TNF-α has been shown to stimulate dopamine transporter (DAT) activity in striatum of C57BL/6 mice by increasing K_m accompanied by reducing the maximum number of DAT (V_{max}) 1 h after TNF-α (Nakajima et al., 2004). The decrease in V_{max} by proinflammatory cytokines is in concurrence with the finding of Lai and colleagues (Lai et al., 2009) showing decreased DAT expression 1 h after peripheral LPS. In concordance, constitutive activation of p40 and p42 MAPK signaling pathways, which are activated by proinflammatory cytokines such as TNF-α, increase DA uptake. However, Moron and colleagues showed that this increase in DAT activity was caused by increased V_{max} without alternating K_m values (Moron et al., 2003). Furthermore, TNF-α increases vesicular monoamine transporter 2 (VMAT-2) activity (Nakajima et al., 2004), implying higher DA vesicle-storage and less DA available for degradation to DOPAC by cytosolic monoamine oxidase. This could explain why DOPAC did not significantly increase during increased DAT-activity. However, the precise role of DAT and VMAT-2 in proinflammatory cytokine-induced anhedonia needs further investigation.

As mentioned before, peripheral LPS increased ICSS thresholds and DA levels in the NAc in rats (Borowski et al., 1998), whereas peripheral IL-2 decreased DA levels in the NAc (Anisman et al., 1996). In the present study, we found a trend towards increased DA levels. Thus, anhedonia, reflected by increased ICSS thresholds, is not necessarily associated with reduced extracellular DA levels in the NAc. Correspondingly, extracellular DA levels in the

NAc are elevated after activation of the HPA axis, indicating that elevations of DA in the NAc are not necessarily associated with hedonia (Wise, 2008). LPS and TNF-α (although less potent than LPS), activate the HPA-axis, whereas IL-2 does not (Dunn, 2000). This could explain why LPS, and to a lesser extent TNF-α, increase DA in the NAc, and IL-2 does not.

In the present experiment ICSS thresholds tended to increase 1 and 4 h after TNF-α, whereas ICSS thresholds returned to baseline 24 h after TNF-α. Furthermore, there was a significant treatment effect, suggesting the presence of anhedonia. This increase was not caused by the inability to turn the wheel because of motor dysfunctions, since TNF-α did not alter response latencies. The TNF-α-induced increase in ICSS thresholds is in agreement with previously reported studies showing that TNF-α increases immobility in the forced swim and tail suspension tests (Kaster et al., 2012), indicating that TNF-α indeed induces depression-like behavior. Furthermore it has been demonstrated that TNF-α injected animals consumed less sucrose or chocolate milk, indicating a state of increased anhedonia (Hayley et al., 1999; Kaster et al., 2012). The measurement of less sweet food consumption, however, might be confounded because TNF-α and other proinflammatory cytokines are known to induce loss of appetite and anorexia (Hayley et al., 1999). In the present study, TNF-α significantly decreased body weight as well. The current ICSS study, however, has the advantage that activity of the reward system is measured directly and is independent of food intake. Nevertheless, one should keep in mind that ICSS only tells us something about the sensitivity of the reward system. Therefore, although reward and pleasure are highly interconnected and hence it is likely that increased ICSS thresholds reflect anhedonia, the latter should be interpreted with caution.

We showed that peripheral administered TNF-α decreased brain reward function, indicating anhedonia, and increased extracellular 5-HIAA and HVA levels in the NAc. This is in agreement with the observation that TNF-α in cerebrospinal fluid is positively correlated with cerebrospinal 5-HIAA and HVA levels in suicide attempters (Lindqvist et al., 2009), suggesting that there is an association between TNF-α-induced changes in monoamine metabolism and anhedonia. We hypothesize that SERT, and possibly DAT, play a role in this process. Indeed, we have recently demonstrated that LPS-induced anhedonia is abolished in SERT knockout rats (van Heesch et al., 2013a). Interestingly, escitalopram (SSRI) reduces circulating TNF-α levels and improves depressive-like behavior in a rat model of post-cardiac infarct depression (Bah et al., 2011). Altogether, this suggests that increased systemic TNF-α levels may play an important role in increased anhedonia, a core symptom of depression. However, further studies are needed to investigate the underlying molecular mechanisms and role of SERT and possibly DAT in TNF-α-induced anhedonia.

4

Lipopolysaccharide increases degradation of central monoamines: An in vivo microdialysis study in the nucleus accumbens and medial prefrontal cortex of mice

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Submitted

Abstract

An increasing amount of evidence suggests that peripheral proinflammatory cytokines contribute to the pathogenesis of depression. Indeed, in rats, peripheral administration of lipopolysaccharide (LPS) increased anhedonia, i.e. the inability to experience pleasure. Recently, we reported that serotonin transporter (SERT) function is required for LPS-induced anhedonia. Less is known, however, about the effect of LPS on the biological activity of dopamine transporters (DAT) and norepinephrine transporters (NET). Therefore, *in vivo* microdialysis was performed in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) of C57BL6/J mice exposed to saline or LPS (133 µg/kg i.p.). To investigate the possible involvement of different monoamine transporters, the triple reuptake inhibitor DOV 216,303 or saline was i.p. injected 30 min before the saline/LPS injection. The dose of LPS, shown to decrease responding for brain stimulation reward in mice, significantly increased extracellular levels of monoamine metabolites (5-HIAA, DOPAC and HVA) in the NAc and mPFC. Remarkably, DOV 216,303 abolished LPS-induced DOPAC and HVA formation in the NAc, suggesting that LPS increases DAT activity in this brain area. LPS also significantly increased DOPAC and HVA formation in the mPFC, where DA reuptake probably takes place via NET, because DAT density is very low in the mPFC. This increase was blocked by DOV 216,303, suggesting that LPS increases prefrontal NET activity as well. Furthermore, DOV 216,303 pretreatment prevented LPS-induced 5-HIAA formation in the mPFC, indicating that LPS increases prefrontal SERT activity. In conclusion, the present findings suggest that peripheral LPS increases DAT activity in the NAc and increases both NET and SERT activity in the mPFC.

Introduction

Since the discovery of reciprocal pathways between immune, endocrine and central nervous system (Ader, 1980), more and more evidence points to a role of proinflammatory cytokines in the development depression (Beumer et al., 2012; Dantzer et al., 2008; Konsman et al., 2002; Miller et al., 2013; Pollak and Yirmiya, 2002). For example, patients with chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and psoriasis have an increased risk to become depressed (Akay et al., 2002; Hauser et al., 2011; Isik et al., 2007; Loftus et al., 2011). These patients have in common that they have increased levels of serum proinflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-α) (Komatsu et al., 2001; Tetta et al., 1990). Moreover, it has been demonstrated that peripheral treatment with interleukin 2 (IL-2) or interferon alpha (IFN-α) highly increases the risk to develop depression in humans (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987).

A core symptom of major depression is anhedonia, i.e. the inability to experience pleasure. In rats, peripherally administered lipopolysaccharide (LPS) reduced brain stimulation reward in the intracranial self-stimulation (ICSS) procedure, a measure for anhedonia (Barr et al., 2003; Borowski et al., 1998; van Heesch et al., 2013a). LPS is an essential component of the outer membrane of gram negative bacteria that activates the immune system and leads to up-regulation of proinflammatory cytokines in both serum and brain (Datta and Opp, 2008; Konsman et al., 2008). Altogether, this suggests that proinflammatory cytokines could induce changes in the brain and alter emotional behavior. However, the underlying mechanisms are largely unknown.

The serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET) critically regulate the duration of cellular actions of serotonin (5-HT), dopamine (DA) and norepinephrine (NE), respectively. Central monoaminergic signaling of 5-HT, DA and NE have been shown to play an important role in emotional behavior and mood disorders. Therefore, monoamines and monoamine transporters, especially 5-HT and SERT, have been studied intensively in major depression (Albert et al., 2012; Prins et al., 2011a). Recently, it has been shown that LPS and proinflammatory cytokines increase SERT activity (Mossner et al., 1998; Zhu et al., 2006; Zhu et al., 2010) and that LPS-induced anhedonia is abolished in SERT knockout rats (van Heesch et al., 2013a), demonstrating that increased SERT function is needed to provoke LPS-induced anhedonia. Whether LPS also increases DAT and NET activity is still under debate.

Elevated monoamine transporter activity does not only lead to increased removal of monoamines from the synaptic cleft, but, especially important for the present study, it also leads to increased metabolism of monoamines. Metabolism of monoamines takes place in the same cells as where the monoamines are produced (Eisenhofer et al., 2004). After

reuptake by monoamine transporters, 5-HT and DA are packed in storage vesicles or metabolized by monoamine oxidase (MAO). In serotonergic neurons, MAO metabolizes 5-HT to 5-hydroxyindole acid aldehyde, and thereafter aldehyde dehydrogenase rapidly metabolizes 5-hydroxyindole acid aldehyde to 5-hydroxyindoleacetic acid (5-HIAA), the most stable metabolite of 5-HT (Deutch and Roth, 2008). DA on the other hand, is metabolized by MAO to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is quickly further metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (Eisenhofer et al., 2004). Thereafter, 5-HIAA and DOPAC diffuse out of the neurons (Deutch and Roth, 2008; Eisenhofer et al., 2004). Subsequently, DOPAC is further metabolized by catechol-*O*-methyl transferase (COMT) to homovanillic acid (HVA), the major metabolite of DA (Eisenhofer et al., 2004). In brain structures where DAT density is low, e.g. the medial prefrontal cortex (mPFC) (Sesack et al., 1998), reuptake of DA takes place via NET (Gresch et al., 1995; Gu et al., 1994). Furthermore, DA can be metabolized in glial cells, thus outside presynaptic neurons. In this case DA is first metabolized by COMT to 3-methoxytyramine (3-MT) followed by oxidation by MAO to HVA. In the latter event the presence of DAT (or NET) is not required for DA metabolism (Eisenhofer et al., 2004). Thus, besides controlling the length of the cellular actions of 5-HT, DA and NE, monoamine transporters also play an important role in the formation of monoamine metabolites. This is supported by a microdialysis study demonstrating that simultaneous inhibition of DAT, SERT and NET by the triple reuptake inhibitor DOV 216,303 (Skolnick et al., 2006) leads to increased extracellular levels of 5-HT, DA and NE, whereas their metabolites 5-HIAA, DOPAC and HVA decreased significantly (Prins et al., 2010; Prins et al., 2011b). Conversely, increased formation of monoamine metabolites may indicate that monoamine transporter function is increased.

The nucleus accumbens (NAc) and mPFC play an important role in the pathophysiology of major depression. It has been hypothesized that the NAc, i.e. the reward center of the brain, and its dopaminergic inputs from the ventral tegmental area (VTA) are crucial for the development of anhedonia (Nestler and Carlezon, 2006). The mPFC, known for its role in overall cognitive functioning and suppression of negative affect (Robbins and Arnsten, 2009), is innervated by dopaminergic neurons from the VTA as well (Williams and Goldman-Rakic, 1998), as well as by serotonergic and noradrenergic fibers arising from the raphe nucleus and locus coeruleus, respectively (Dillier et al., 1978; Gold and Chrousos, 2002; Molliver, 1987). Furthermore, DAT density in the PFC is very low (Sesack et al., 1998), and therefore, prefrontal DA is predominantly metabolized by COMT in glial cells (Yavich et al., 2007) or taken up by noradrenergic cells via norepinephrine transporters, where MAO metabolizes DA to DOPAC (Gresch et al., 1995; Gu et al., 1994). Altogether, this makes both the NAc and mPFC interesting brain areas to analyze LPS-induced alterations in extracellular levels of 5-HT, 5-HIAA, DA, DOPAC and HVA, and consequently SERT, DAT and NET function.

First, we investigated whether LPS is able to decrease brain stimulation reward in the ICSS procedure in mice. Furthermore, two microdialysis experiments were performed to investigate whether and how LPS affects monoamine transporter function in the NAc and mPFC of mice. In the first study, the effects of LPS on DA, DOPAC and HVA levels and on 5-HT and 5-HIAA levels were measured. In the second study, it was investigated whether blockade of DAT, SERT and NET by the triple reuptake inhibitor DOV 216,303 (Skolnick et al., 2006) could prevent the LPS-induced effects on extracellular monoamine and monoamine metabolite levels in the NAc and mPFC.

Methods

Animals

Fifty-four male C57BL/6J mice (Charles River, Maastricht) arrived at the age of 9 to 10 weeks and were socially housed, eight to ten mice per cage on a 12 h light/dark cycle with lights on at 6:00 am and off at 6:00 pm. Food and water were available *ad libitum*. Mice had one week to acclimate to their new environment. Studies were conducted according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and were approved by the Ethical Committee for Animal Research of Utrecht University, The Netherlands. During the experiments all efforts were made to minimize animal pain, distress and discomfort.

Treatment

Pretreatment: DOV 216,303

The triple reuptake inhibitor DOV 216,303 [(±)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride] (Sepracor Inc., Marlborough, USA) was dissolved in saline and prepared freshly on test days (0.5 mg/ml). DOV 216,303 was administered intraperitoneally (i.p.), 5 mg/kg in a volume of 10 ml/kg. This dose was based on observations described earlier in mice (Caldarone et al., 2010). Control animals received i.p. injections of saline in a volume of 10 ml/kg.

Treatment: LPS

Escherichia Coli derived lipopolysaccharide (LPS) (Sigma, 0127:B8) was dissolved in saline and prepared freshly on test days from the stock solution (0.5 mg/kg dissolved in saline, stored at –80 ^{o}C). 133.33 µg/kg LPS was administered i.p. in a volume of 10 ml/kg. Control animals received i.p. injections of saline in a volume of 10 ml/kg.

Brain stimulation reward

ICSS surgery

Eight mice were anesthetized by inhalation of isoflurane gas (2%), mixed with oxygen and placed in a stereotaxic instrument. Lidocaine hydrochloride (2%) + adrenaline (0.001%) were applied in the incision. Twisted bipolar electrodes (cut to 6 mm (Bilaney Consultants GmbH)) were implanted in the lateral hypothalamus (anterioposterior –0.5 mm; mediolateral +/– 1.3 mm (under a 0^{o} angle) from bregma; dorsoventral –5.0 mm from skull surface (G. Paxinos, 2001)). Electrodes were fixed with three screws and dental acrylic on the skull. All animals received the non-steroidal anti-inflammatory drug (NSAID) Rimadyl for pain relief (5 mg/kg, subcutaneously, twice a day, four injections in total). After surgery, animals were housed two per cage.

ICSS

One week after surgery mice were trained twice a day in the ICSS paradigm according to the discrete-trial current-intensity procedure as described before by Kornetsky (1979) and Kenny et al. (2003). Each session consisted of four alternating series of descending and ascending current intensities starting with a descending series. A set of five trials was presented for each current intensity, and the intensity was altered in 5 µA steps between blocks of trials. The initial current intensity was set approximately 40 µA above baseline thresholds so that the initial current intensity of each subject was within 50 to 200 µA. A one-quarter turn of the wheel manipulandum within 7.5 sec after the presentation of a non-contingent electrical stimulation (positive response) resulted in another electrical stimulus. The time to respond to the non-contingent electrical stimulus was recorded and called the response latency. After a positive or negative response (no turn of the wheel within 7.5 sec after the non-contingent stimulus) the inter-trial interval started with an average duration of 10 sec (7.5-12.5 sec range) followed by another trial with the delivery of a non-contingent stimulus. The onset of the next trial was delayed in case mice responded during the inter-trial interval. After the establishment of steady ICSS thresholds (3 consecutive days during which ICSS thresholds varied by <10%), mice received an i.p. injection of either saline or

LPS (133.33 µ/kg) and were tested in the ICSS paradigm 1, 4, 24, 48, 72 and 96 h after injection. Three weeks after the first injection, the experiment was repeated, as animals were tested in a cross-over design in such a way that all the animals received a saline (n = 8) and LPS (n = 8) injection. During each measurement ICSS thresholds and response latencies were recorded. ICSS thresholds and response latencies obtained during the three baseline measurements were used to calculate percentage change from baseline values at each time point tested after the injections. After the last measurement, animals were sacrificed.

Body weight

Body weight of mice was measured daily around noon. Body weight measured during the three ICSS baseline days was averaged and used to calculate body weight at baseline and delta body weight values at different time points after saline or LPS injection.

Microdialysis

Microdialysis surgery

Mice were anesthetized by inhalation of a mixture of isoflurane gas (2%) and oxygen and placed in a stereotaxic instrument. The microdialysis study comprised two consecutive experiments. In the first microdialysis study mice were pretreated with saline. In half of the saline pretreated animals, cuprofane microdialysis probes (MAB 4.6.1 CU, 1 mm membrane length) were implanted in the nucleus accumbens (NAc) (n = 14), whereas in the other half of the saline pretreated animals probes (MAB 4.6.2. CU, 2 mm membrane length) were implanted in the medial prefrontal cortex (mPFC) (n = 16). In the other microdialysis study mice were pretreated with the triple reuptake inhibitor DOV 216,303. In this experiment two probes per mouse were implanted; one probe in the NAc, the other in the mPFC (n =16). The coordinates of the NAc and mPFC were anterioposterior +1.5 mm; mediolateral +0.8 mm (under a 0^{o} angle) from bregma; dorsoventral –5.0 mm from skull surface and anterioposterior +1.9 mm; mediolateral +0.9 mm (under a 8^{o} angle) from bregma; dorsoventral –3.3 mm from skull surface, respectively (G. Paxinos, 2001). Probes were anchored with nonacrylic dental cement on the scull. After implantation of the microdialysis probes, mice were housed individually and placed in the microdialysis room until the end of the experiment.

Microdialysis study

The microdialysis studies were performed in conscious freely moving mice, one day after implantation of the microdialysis probes. A pump (KdScientific Pump 220 series, USA) perfused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant flow rate of 0.02 ml/h. During microdialysis, the flow rate was set at 0.07 ml/h. At 8:00 am mice were connected to a channel swivel (type 375/D/22QM) which allowed them to move freely. Three h after connection, 30-minute samples were manually collected in vials containing 11.7 µl of 0.1 M acetic acid and frozen at –80 ^{o}C until analysis with HPLC. From 11:00 am until 1:00 pm four baseline samples were collected. Subsequently, animals in the saline pretreatment microdialysis study were i.p. injected with saline (NAc: n = 14; mPFC: n = 16), whereas animals in the DOV 216,303 pretreatment microdialysis study were i.p. injected with 5 mg/kg DOV 216,303 (NAc and mPFC: n = 16). Thirty minutes later, half of the animals in each pretreatment group received an i.p. saline injection, whereas the other half of each group received an i.p. LPS injection (NAc saline pretreatment: n = 7, mPFC saline pretreatment: n = 8 and NAc and mPFC DOV 216,303 pretreatment: n = 8, respectively). After the last injection samples were collected for an additional 4 h. In each microdialysis study 13 30-minute samples were collected in total. Immediately after collection of the last microdialysis sample animals were sacrificed. The brains were dissected and stored in formaldehyde to verify probe localization later on.

HPLC

Microdialysis samples were stored at –80 °C until analysis. Neurotransmitters, dopamine (DA) and serotonin (5-HT) and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) respectively were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, The Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 x 50 mm, 3 µm particle size) in combination with detector cell 1, separated and detected DA and 5-HT. Column 2 (NeuroSep 115 C18 1 x 150 mm, 3 µm particle size) in combination with detector cell 2, separated and detected the metabolites. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 18.5 % Methanol and 400 mg/L OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5 % methanol and 700 mg/L OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. The neurotransmitters were detected electrochemically using µVT-03 flow cells (Antec) with glassy carbon working electrodes.

Potential settings were for DA and 5-HT +0.30 V versus Ag/AgCl and for the metabolites +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec). The limit of detection was 0.05 nM (S/N ratio 3:1).

Histology

Two days before brain slicing, the brains were transferred from formaldehyde to a 30% sucrose solution. Probe placements were verified on 60 µm cresyl violet stained sections obtained with the frozen technique. Data were discarded when the microdialysis probe was not placed in the NAc or mPFC.

Statistical analysis

Baseline body weight, baseline ICSS thresholds and baseline ICSS response latencies were analyzed with use of independent *t*-tests to exclude differences in baseline between the two groups.

Body weight data is expressed as the difference from baseline data (delta body weight), whereas ICSS current-thresholds data and response latency data are expressed as a percentage of the baseline data. Delta body weight was analyzed by repeated measures analysis of variance (repeated measures ANOVA) with time (6 levels: 0h, 1h, 24h, 48h, 72h and 96h) as within subject factor and treatment (saline or LPS) as between subject factor. ICSS current-thresholds and response latencies were analyzed by repeated measures ANOVA with time (7 levels: 0h, 1h, 4h, 24h, 48h, 72h and 96h) as within subject factor and treatment (saline or LPS) as between subject factor. In case of a significant time x treatment interaction, effects of treatment on individual time points were analyzed with post hoc *t*-tests with treatment (saline or LPS) as grouping variable.

Mean NAc and mPFC baseline monoamine (metabolite) levels in the saline pretreatment group and mean NAc and mPFC baseline monoamine (metabolite) levels in the DOV 216,303 pretreatment group were analyzed with use of independent *t*-tests. Then, measurements were expressed as a percentage of baseline and analyzed by repeated measures ANOVA with time (4 levels: –120 min, –90 min, –60 min and –30 min) as within subject factor and treatment (saline or LPS) as between subject factor to exclude differences between groups at baseline. Subsequently, post-injection data was compared in a repeated measures ANOVA with time (9 levels: 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min and 240 min) as within subject factor and treatment (saline or LPS) as between subject factor. In case of a significant time x treatment interaction, effects of LPS on individual time points were analyzed with post hoc *t*-tests with treatment (saline or LPS) as the grouping variable. When the assumption of sphericity was violated, the results were

corrected by the Greenhouse-Geisser procedure. Corresponding area under the curve (AUC) values were compared with use of independent *t*-tests with treatment as the grouping variable (saline or LPS). All data were analyzed using the SPSS 20 software statistical package.

Results

Brain stimulation reward

Thresholds

Before injections, mean absolute baseline ICSS thresholds (± S.E.M.) were 91.55 ± 19.31 µA and 86.25 ± 15.69 µA in the saline and LPS group, respectively, and did not significantly differ between groups. Repeated measures ANOVA revealed a significant time x treatment interaction and significant effect of treatment ($F(1.7,24.0) = 6.263$, $p = 0.009$, $\varepsilon = 0.286$ and $F(1,14) = 9.2$, $p = 0.009$, respectively) (***Figure 4.1: A***). Post hoc *t*-tests per time point showed significant increases in ICSS thresholds 1 h and 4 h after LPS injection.

Response latencies

Mean baseline ICSS response latencies (± S.E.M.) were 2.58 ± 0.53 s and 2.58 ± 0.31 s in saline and LPS treated mice, respectively, and did not significantly differ between groups. Repeated measures ANOVA showed a significant time x treatment interaction ($F(6,84) = 2.8$, $p = 0.015$) and a trend towards an effect of treatment ($F(1,14) = 3.3$, $p = 0.091$) (***Figure 4.1: B***). Post hoc *t*-tests per time point revealed significant decreases in ICSS response latencies 1 h and 4 h after exposure to LPS.

Body weight

Three days before saline and LPS injections mean body weights (± standard error of the mean (S.E.M.)) were 31.50 ± 0.77 g and 32.00 ± 0.80 g, respectively, and did not differ significantly between groups. Repeated measures ANOVA revealed a significant time x treatment interaction and significant effect of treatment ($F(2.2,31.3) = 12.6$, $p < 0.001$, $\varepsilon =$

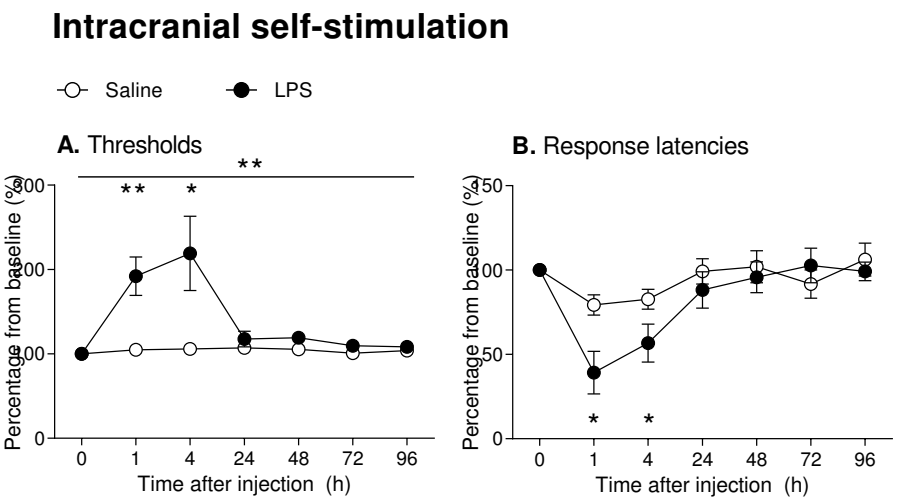

Figure 4.1: The effect of LPS on ICSS thresholds in mice. ICSS thresholds and response latencies were measured 1, 4, 24, 48, 72 and 96 h after exposure to saline or LPS. ICSS thresholds (**A**) and response latencies (**B**) are presented as percentage change from baseline values. In both figures values are presented as mean ± S.E.M.; statistically significant results are indicated as * $p < 0.05$, ** $p < 0.01$.

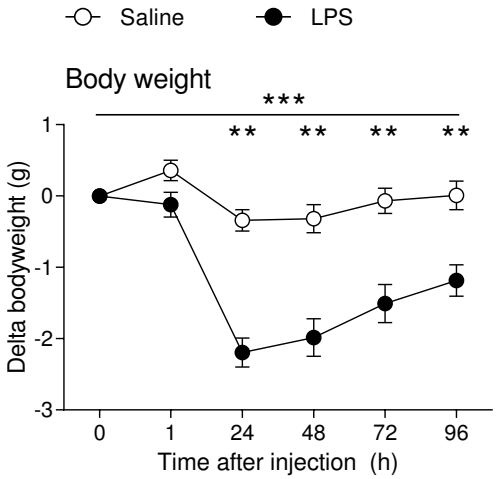

Figure 4.2: The effect of LPS on body weight in mice. Body weight was measured 1, 24, 48, 72 and 96 h after exposure to saline or LPS. Delta bodyweight is presented as mean ± standard error of the mean. Statistically significant results are indicated as ** $p < 0.01$, *** $p < 0.001$.

0.448 and $F(1,14) = 35.0$, $p < 0.001$, respectively) (***Figure 4.2***). Post hoc *t*-tests per time point showed significant decreases in body weight from 24 h till 96 h after exposure to LPS.

Microdialysis

Probe placement in the NAc and mPFC

In total 4 animals were excluded because of incorrect microdialysis probe placement in the NAc. Therefore, the saline-saline, saline-LPS, DOV-saline and DOV-LPS group consisted of 6, 6, 6 and 8 animals, respectively. Because in 2 animals DOPAC levels were too high to be measured by HPLC, these animals were excluded from the analysis of microdialysis data of DOPAC levels in the NAc. Therefore, for DOPAC, the saline-saline, saline-LPS, DOV-saline and DOV-LPS group consisted of 6, 6, 5 and 7 mice, respectively.

Two mice were excluded from data analysis due to wrong probe placements in the mPFC. Consequently, 8, 7, 7 and 8 mice were included for data analysis in the saline-saline, saline-LPS, DOV-saline and DOV-LPS group, respectively.

Baseline monoamine and monoamine metabolite levels in the NAc and mPFC

With the exception of prefrontal 5-HIAA levels in the saline pretreatment microdialysis study, absolute mean baseline values of DA, DOPAC, HVA, 5-HT and 5-HIAA did not differ between groups in the 2 microdialysis studies (***Table 4.1***). Furthermore, repeated measure ANOVAs for the first four baseline measurements revealed that percentage change from baseline DA, DOPAC, HVA, 5-HT and 5-HIAA values did not differ between groups in the saline pretreatment microdialysis study as well as in the DOV 216,303 pretreatment microdialysis study. In both the NAc and mPFC there were no significant time x treatment interactions nor significant effects of treatment. Thus, before exposure to DOV 216,303 or LPS all groups were comparable.

The effect of DOV 216,303 on LPS-induced alterations in DA, DOPAC and HVA in the NAc and mPFC

LPS did not affect DA levels in the NAc. Repeated measures ANOVA for DA showed neither a significant time x treatment interaction nor an effect of treatment (trend) (***Figure 4.3: A***). Once animals were pretreated with DOV 216,303, DA levels in the NAc still did not differ between saline and LPS treated mice (***Figure 4.3: B***). DA AUC values confirmed that there was no significant difference between saline and LPS treated mice in both the saline

	Saline pretreatment microdialysis study					
	Nucleus accumbens			Medial prefrontal cortex		
	Saline	LPS	*p*-value	Saline	LPS	*p*-value
DA	1.10 ± 0.32	0.94 ± 0.43	NS	0.18 ± 0.02	0.15 ± 0.02	NS
DOPAC	143.2 ± 42.1	120.7 ± 18.2	NS	36.2 ± 8.2	19.4 ± 2.3	NS
HVA	111.5 ± 30.3	110.6 ± 9.9	NS	77.3 ± 12.8	54.8 ± 6.4	NS
5-HT	0.12 ± 0.01	0.10 ± 0.01	NS	0.13 ± 0.02	0.12 ± 0.01	NS
5-HIAA	57.2 ± 5.3	61.5 ± 4.2	NS	80.2 ± 6.3	60.3 ± 1.6	$p < 0.05$
	DOV 216,303 pretreatment microdialysis study					
	Nucleus accumbens			Medial prefrontal cortex		
	Saline	LPS	*p*-value	Saline	LPS	*p*-value
DA	1.19 ± 0.11	1.17 ± 0.12	NS	0.32 ± 0.05	0.25 ± 0.03	NS
DOPAC	271.4 ± 52.2	240.9 ± 44.5	NS	20.0 ± 2.1	31.3 ± 5.4	NS
HVA	275.5 ± 39.6	248.6 ± 32.4	NS	74.6 ± 5.3	95.5 ± 10.3	NS
5-HT	0.08 ± 0.04	0.06 ± 0.01	NS	0.13 ± 0.02	0.13 ± 0.03	NS
5-HIAA	118.6 ± 10.0	97.6 ± 4.1	NS	116.3 ± 9.2	119.6 ± 10.3	NS

Table 4.1: Baseline monoamine and monoamine metabolite levels in the NAc and mPFC. Accumbal and prefrontal absolute mean baseline monoamine and monoamine metabolite levels (± standard error of the mean), thus before exposure to DOV 216,303 or LPS, in the saline pretreatment microdialysis study and DOV 216,303 pretreatment microdialysis study are presented. Statistically significant results are indicated as $p < 0.05$. Results that are not significant are indicated as NS.

pretreatment and DOV 216,303 pretreatment microdialysis study (***Figure 4.3: C***). In contrast to what was found in the NAc, repeated measures ANOVA revealed a significant time x treatment interaction ($F(3.5,44.9) = 14.9$, $p < 0.001$, $\varepsilon = 0.431$) and significant effect of treatment ($F(1,13) = 37.4$, $p < 0.001$) for extracellular DA levels in the mPFC (***Figure 4.5: A***). Post hoc *t*-tests per time point showed that LPS significantly increased extracellular DA levels from 60 min till 240 min after exposure to LPS (at least $p < 0.05$). After pretreatment with DOV 216,303 repeated measures ANOVA revealed a significant time x treatment interaction ($F(2.2,28.5) = 3.2$, $p = 0.050$, $\varepsilon = 0.274$), without an effect of treatment (trend) (***Figure 4.5: B***). Post hoc *t*-tests per time point showed that DA levels in the DOV 216,303 pretreatment microdialysis study were significantly higher in LPS treated animals compared to saline treated animals from 120 min till 240 min after exposure to LPS (at least $p < 0.05$). Furthermore LPS significantly increased AUC values of DA in the mPFC in the saline pretreatment microdialysis study ($p < 0.001$), but not in the DOV 216,303 pretreatment microdilaysis study (trend) (***Figure 4.5: C***).

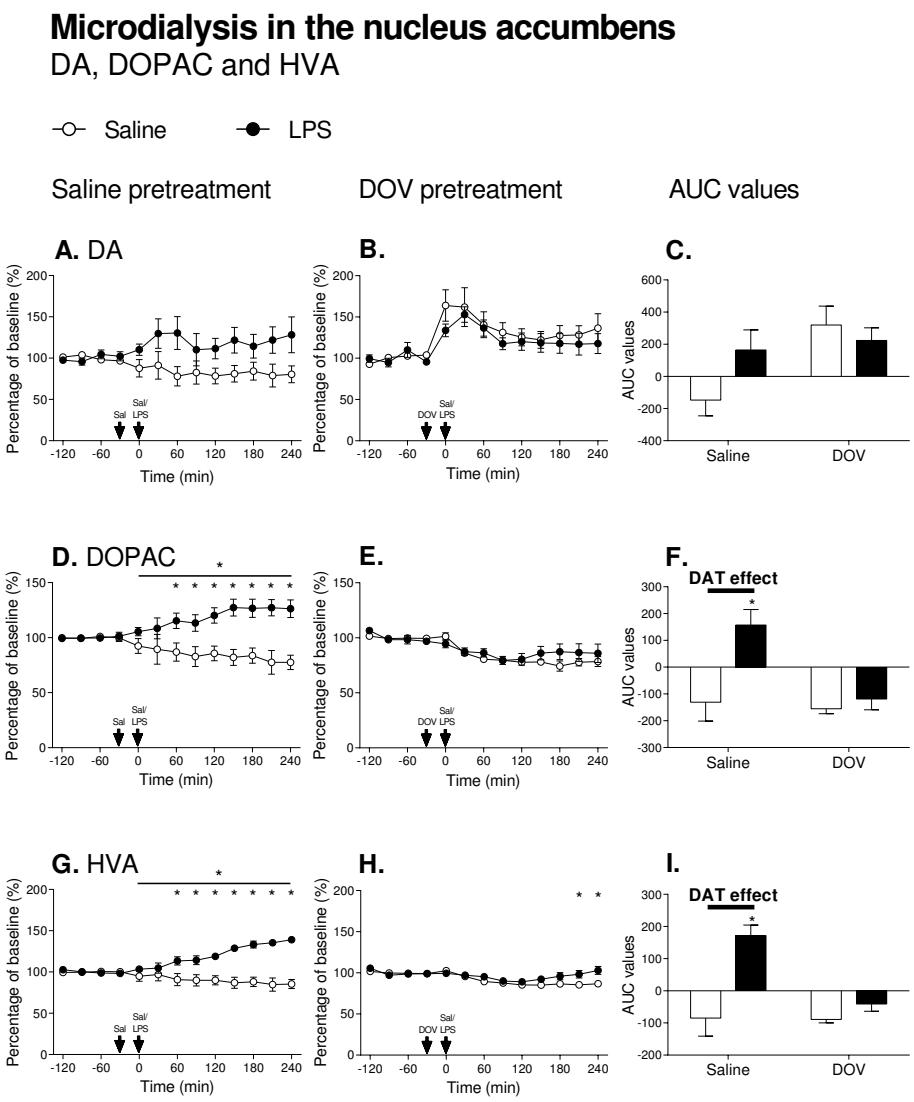

Figure 4.3: Microdialysis in the NAc: DA, DOPAC and HVA. The effect of DOV 216,303 on LPS-induced changes in DA, DOPAC and HVA in the NAc. DA, DOPAC and HVA levels were measured under basal condition and after pre-exposure to saline (first column: DA: **A**, DOPAC: **D** and HVA: **G**) or DOV 216,303 (second column; DA: **B**, DOPAC: **E** and HVA: **H**) (time point -30 min) followed by a saline (white bullets) or LPS injection (black bullets) (time point 0 min). Percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated mice at a single time point are indicated as * $p < 0.05$. Corresponding mean area under the curve (AUC) values ± standard error of the mean are shown in the third column (DA: **C**, DOPAC: **F** and HVA: **I**). For AUC values, statistically significant differences between two groups are indicated as * $p < 0.05$.

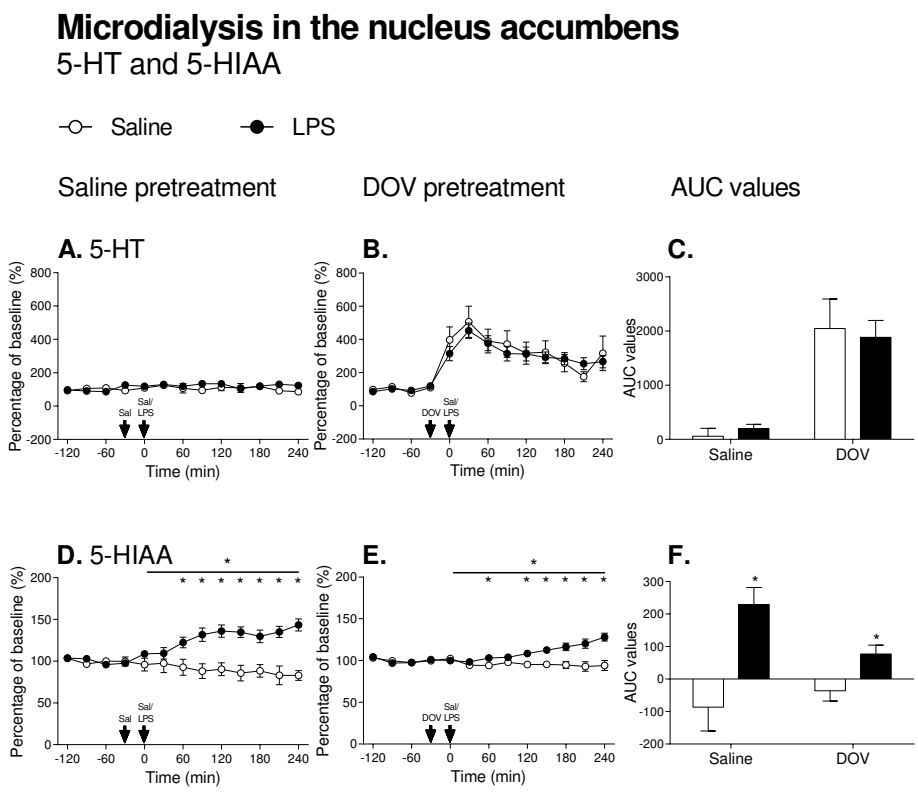

Figure 4.4: Microdialysis in the NAc: 5-HT and 5-HIAA. The effect of DOV 216,303 on LPS-induced changes in 5-HT and 5-HIAA in the NAc. 5-HT and 5-HIAA levels were measured under basal condition and after pre-exposure to saline (first column: 5-HT: **A** and 5-HIAA: **D**) or DOV 216,303 (second column; 5-HT: **B** and 5-HIAA: **E**) (time point -30 min) followed by a saline (white bullets) or LPS injection (black bullets) (time point 0 min). Percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated mice at a single time point are indicated as * $p < 0.05$. Corresponding mean area under the curve (AUC) values ± standard error of the mean are shown in the third column (5-HT: **C** and 5-HIAA: **F**). For AUC values, statistically significant differences between saline and LPS-treated mice are indicated as * $p < 0.05$.

In both the NAc and mPFC, DOPAC levels increased significantly in response to LPS (***Figure 4.3: D*** and ***Figure 4.5: D***, respectively). There was a significant time x treatment interaction (NAc: $F(3.4,33.6) = 8.3$, $p < 0.001$, $\varepsilon = 0.420$ and mPFC: $F(2.1,27.5) = 37.0$, $p < 0.001$, $\varepsilon = 0.264$) and significant effect of treatment (NAc: $F(1,10) = 10.6$, $p = 0.009$ and mPFC: $F(1,13) = 33.1$, $p < 0.001$). In both brain areas, DOPAC levels were significantly increased 60 min after exposure to LPS till the end of the study (at least $p < 0.05$). Pre-exposure to DOV 216,303 completely abolished the LPS-induced increases in DOPAC in

the NAc (***Figure 4.3: E***). There was neither a time x treatment interaction, nor an effect of treatment. In the mPFC, on the other hand, there was a significant time x treatment interaction ($F(2.1,27.1) = 16.7$, $p < 0.001$, $\varepsilon = 0.261$) without an effect of treatment (trend) (***Figure 4.5: E***). Post hoc *t*-tests per time point only revealed a significant increase in DOPAC levels at 150 min till 240 min after exposure to LPS (at least $p < 0.05$). In the saline pretreatment microdialysis study AUC values were significantly higher in LPS treated mice compared to saline treated mice in both the NAc ($p = 0.010$) and mPFC ($p = 0.001$) (***Figure 4.3: F*** and ***Figure 4.5: F***, respectively). Pretreatment with DOV 216,303, however, abolished the LPS-induced increases in DOPAC AUC values in both brain areas.

HVA, the other metabolite of DA, also increased significantly after exposure to LPS in both brain areas (NAc: ***Figure 4.3: G*** and mPFC: ***Figure 4.5: G***). Repeated measures ANOVAs revealed significant time x treatment interactions (NAc: $F(8,80) = 38.3$, $p < 0.001$ and mPFC: $F(1.8,23.4) = 48.7$, $p < 0.001$, $\varepsilon = 0.225$) and significant effects of treatment (NAc: $F(1,10) = 17.4$, $p = 0.002$ and mPFC: $F(1,13) = 47.5$, $p < 0.001$). In the NAc as well as in the mPFC HVA levels were significantly increased from 60 min after exposure to LPS until the end of the study (at least $p < 0.05$). Although repeated measures ANOVAs revealed significant time x treatment interactions in both brain areas (NAc: $F(1.4,17,3) = 4.5$, $p = 0.036$, $\varepsilon = 0.180$ and mPFC: $F(2.8,28.3) = 7.0$, $p = 0.003$, $\varepsilon = 0.272$), DOV 216,303 inhibited the LPS effect on extracellular HVA levels (NAc: ***Figure 4.3: H*** and mPFC: ***Figure 4.5: H***). In the NAc (trend) as well as in the mPFC there was no significant effect of treatment. Furthermore did post hoc *t*-tests per time point reveal that LPS-induced increases in HVA levels were almost completely abolished by pre-exposure to DOV 216,303. In both brain areas HVA was only significantly elevated in LPS-treated animals at the end of the microdialysis study (t = 210 and 240 min) (at least $p < 0.05$). The observation that DOV 216,303 pretreatment inhibits LPS-induced HVA formation is supported by the observation that HVA AUC values of LPS treated animals in the saline pretreatment microdialysis study were significantly increased (NAc: $p = 0.003$ and mPFC: $p < 0.001$), whereas HVA AUC values of LPS treated animals in the DOV 216,303 pretreatment microdialysis study did not differ from HVA AUC values of saline treated mice in the NAc (trend) as well as in the mPFC (NAc: ***Figure 4.3: I*** and mPFC: ***Figure 4.5: I***).

The effect of DOV 216,303 on LPS-induced alterations in 5-HT and 5-HIAA in the NAc and mPFC

In both the NAc and mPFC, LPS did not affect extracellular 5-HT levels (***Figure 4.4: A*** and ***Figure 4.6: A***, respectively). For both brain areas repeated measures ANOVAs showed neither a time x treatment interaction nor an effect of treatment. Also after pretreatment with DOV 216,303, repeated measures ANOVA still revealed neither a time x treatment

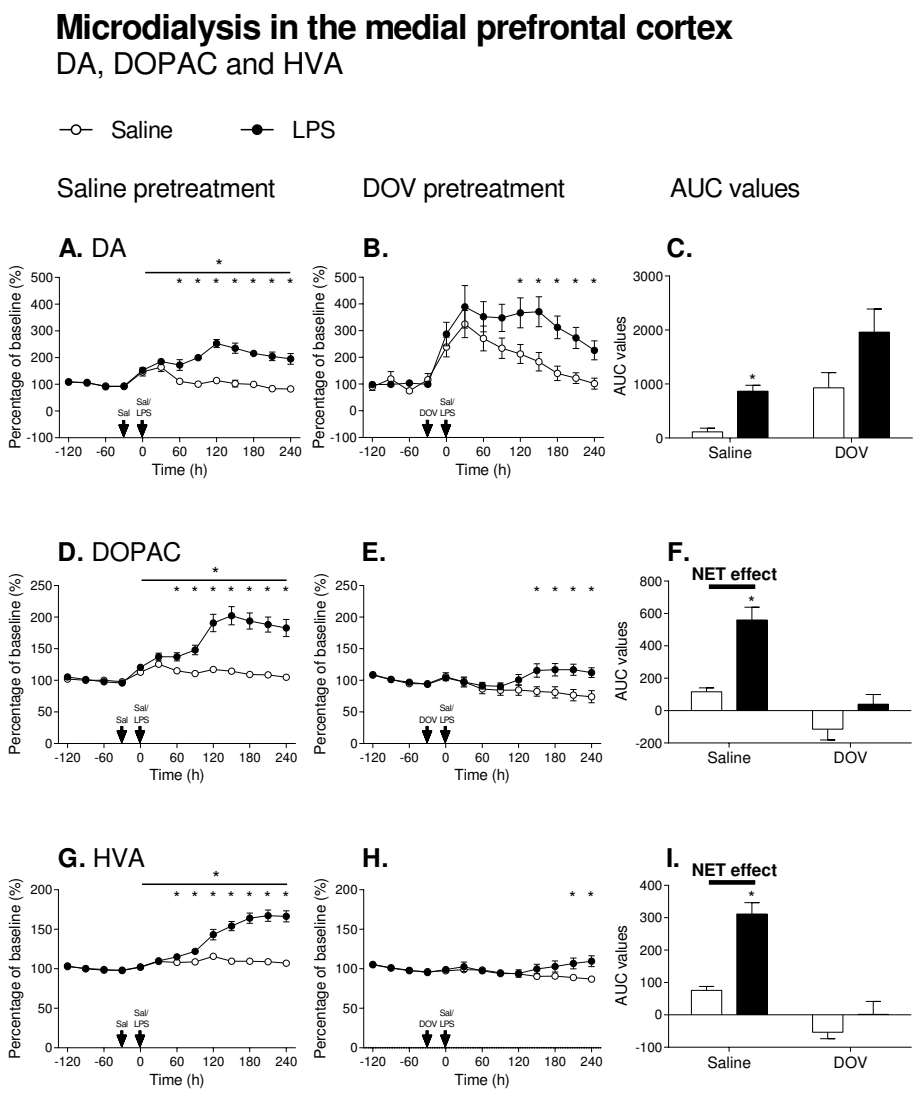

Figure 4.5: Microdialysis in the mPFC: DA, DOPAC and HVA. The effect of DOV 216,303 on LPS-induced changes in DA, DOPAC and HVA in the mPFC. DA, DOPAC and HVA levels were measured under basal condition and after pre-exposure to saline (first column: DA: **A**, DOPAC: **D** and HVA: **G**) or DOV 216,303 (second column; DA: **B**, DOPAC: **E** and HVA: **H**) (time point -30 min) followed by a saline (white bullets) or LPS injection (black bullets) (time point 0 min). Percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated mice at a single time point are indicated as * $p < 0.05$. Corresponding mean area under the curve (AUC) values ± standard error of the mean are shown in the third column (DA: **C**, DOPAC: **F** and HVA: **I**). For AUC values, statistically significant differences between saline and LPS-treated mice are indicated as * $p < 0.05$.

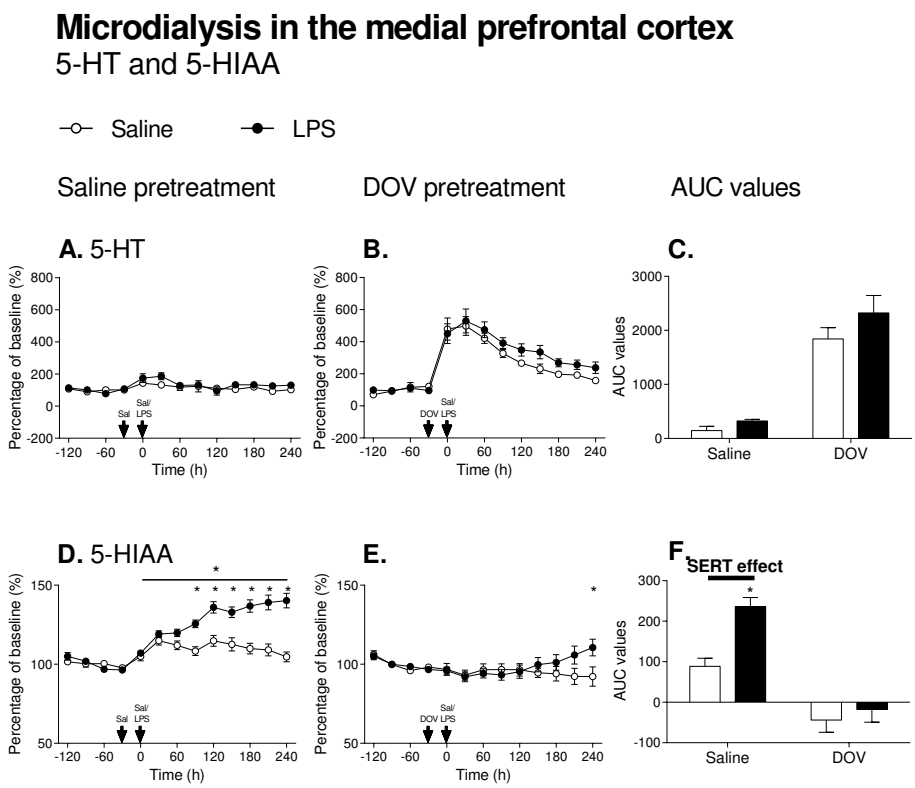

Figure 4.6: Microdialysis in the mPFC: 5-HT and 5-HIAA. The effect of DOV 216,303 on LPS-induced changes in 5-HT and 5-HIAA in the mPFC. 5-HT and 5-HIAA levels were measured under basal condition and after pre-exposure to saline (first column: 5-HT: **A** and 5-HIAA: **D**) or DOV 216,303 (second column; 5-HT: **B** and 5-HIAA: **E**) (time point -30 min) followed by a saline (white bullets) or LPS injection (black bullets) (time point 0 min). Percentage change from baseline data was presented as mean ± standard error of the mean. Statistically significant results between saline and LPS treated mice at a single time point are indicated as * $p < 0.05$. Corresponding mean area under the curve (AUC) values ± standard error of the mean are shown in the third column (5-HT: **C** and 5-HIAA: **F**). For AUC values, statistically significant differences between two groups are indicated as * $p < 0.05$.

interaction nor an effect of treatment (NAc: ***Figure 4.4: B*** and mPFC: ***Figure 4.6: B***). Corresponding AUC values confirmed that LPS did not affect extracellular levels of 5-HT in saline-pretreated and DOV 216,303-pretreated mice in both brain areas (NAc: ***Figure 4.4: C*** and mPFC: ***Figure 4.6: C***).

In contrast to 5-HT, LPS led to a significant increase in its metabolite 5-HIAA (NAc: ***Figure 4.4: D*** and mPFC: ***Figure 4.6: D***). Repeated measures ANOVA revealed that there was a significant time x treatment interaction (NAc: $F(4.2,42.0) = 16.1$, $p < 0.001$, $\varepsilon = 0.525$ and mPFC: $F(3.6,47.1) = 13.2$, $p < 0.001$, $\varepsilon = 0.452$) and significant effect of treatment (NAc: $F(1,10) = 13.6$, $p = 0.004$ and mPFC: $F(1,13) = 26.2$, $p < 0.001$) in both the NAc and mPFC. Post hoc *t*-tests showed that 5-HIAA levels were significantly increased 60 min and 90 min after exposure to LPS in the NAc and mPFC, respectively. This increase was still observed at the end of the study, i.e. 240 min after exposure to LPS (at least $p < 0.05$). Pre-exposure to DOV 216,303 did not prevent LPS-induced increases in extracellular 5-HIAA levels in the NAc. There was a significant time x treatment interaction ($F(2.4,29.0) = 19.0$, $p < 0.001$, $\varepsilon = 0.302$) and a significant effect of treatment ($F(1,12) = 8.3$, $p = 0.014$) (***Figure 4.4: E***). Post hoc *t*-tests per time point showed significant increases in 5-HIAA at 60 min after exposure to LPS and from 120 min after exposure to LPS till the end of the study (at least $p < 0.05$). In the mPFC, however, there was a significant time x treatment interaction ($F(2.2,28.1) = 7.6$, $p = 0.002$, $\varepsilon = 0.271$), without a significant effect of treatment (***Figure 4.6: E***). Interestingly, post hoc *t*-tests per time point revealed that 5-HIAA levels were only elevated at the end of the study (t = 240 min) (at least $p < 0.05$). Also AUC values show that LPS led to a significant increase in 5-HIAA AUC values in both brain areas (NAc: $p = 0.005$ and mPFC: $p < 0.001$), whereas after pretreatment with DOV 216,303 LPS led to a significant increase in 5-HIAA AUC values in the NAc ($p = 0.019$) (***Figure 4.4: F***) but not in the mPFC (***Figure 4.6: F***).

Discussion

Here it is shown that peripherally administered LPS significantly decreases brain stimulation reward in mice, reflecting anhedonia, i.e. the inability to experience pleasure. Furthermore, we showed that LPS increased extracellular monoamine metabolite levels in the NAc and mPFC, which was completely, or at least partly, prevented by pretreatment with the triple reuptake inhibitor DOV 216,303. In this manner, we indirectly showed that LPS increased DAT activity in the NAc and both SERT and NET activity in the mPFC.

LPS decreases brain stimulation reward, reflecting anhedonia.

The observed LPS-induced reductions in brain stimulation reward are in agreement with earlier ICSS studies performed in rats (Barr et al., 2003; Borowski et al., 1998; van Heesch et al., 2013a). Although LPS is known to decrease locomotor activity (Dunn and Swiergiel, 2005), response latency data revealed that LPS-treated mice responded even faster to the

noncontingent stimulus compared to saline-treated mice. This demonstrates that reductions in brain stimulation reward were not caused by inability to turn the wheel due to reductions in locomotor activity. Furthermore, ICSS has the advantage that activity of the reward system is measured directly and therefore is independent of food intake (Spies, 1965). Therefore, the LPS-induced reductions in body weight in the present study probably did not affect responding in the ICSS procedure, suggesting that increased ICSS thresholds represent anhedonia.

LPS increases DAT activity in the NAc

LPS significantly increased DOPAC and HVA levels in the NAc. Recently, we have shown that LPS increased extracellular HVA levels in the NAc of rats as well (van Heesch et al., unpublished results). Furthermore, it has been shown that extracellular levels of HVA, and to a lesser extent DOPAC, increased significantly after exposure to TNF-alpha or IL-1 in combination with a mild stressor (Merali et al., 1997; van Heesch et al., 2013b). LPS-induced elevations of extracellular HVA demonstrates that metabolism of DA and/or DOPAC by COMT is increased. Indeed, in a microdialysis study in the NAc and mPFC of COMT knockout mice, HVA was not detectable, showing that COMT is necessary to produce HVA (Kaenmaki et al., 2010). The observation that DOV 216,303 abolished the LPS-induced increases in DOPAC and HVA in the NAc (AUC values) suggests that LPS increases DAT function. This is in correspondence with a previous study, which ascribed TNF-α to increase striatal DAT function (Nakajima et al., 2004). Furthermore, another group showed that activation of p40 and p42 MAPK signaling pathways, which are known to be activated by LPS and proinflammatory cytokines (Sheng et al., 2011), increased DA uptake by increasing V_{max} without alternating K_m values (Moron et al., 2003). On the other hand, it has also been reported that LPS decreases striatal DAT expression (Lai et al., 2009) and that uptake of DA is not affected in midbrain synaptosomes of mice peripherally injected with LPS (Zhu et al., 2010). Nevertheless, together with the observations in this report, most data indicate that LPS increases DAT function. However, further investigations are needed to unravel the underlying molecular mechanisms.

LPS increases NET activity in the mPFC

Interestingly, DOPAC and HVA levels also increased significantly in the mPFC in response to LPS despite of very low DAT density in this brain area (Sesack et al., 1998). Probably significant increases in prefrontal DOPAC are still possible because DA has a high affinity for NET as well (Gresch et al., 1995; Gu et al., 1994), suggesting that LPS increases NET function as well. Indeed, we showed that pre-exposure to DOV 216,303 prevented the LPS-induced formation of DOPAC and HVA in the mPFC (AUC values).

This suggests that increased activity of NET is necessary for the LPS-induced formation of DOPAC and HVA in this brain structure. This presumption is in correspondence with the observation that i.p. administration of LPS or IL-1 leads to increased levels of 3-methoxy-4-hydroxyphenylglycol (MHPG), i.e. the major metabolite of NE, in the mPFC (Dunn, 2006; Merali et al., 1997). Furthermore, it has been demonstrated that peripheral injection of LPS induces reuptake of NE in mouse brain (Zhu et al., 2010). Altogether this suggests that LPS increases NET function in the mPFC as well.

LPS increases SERT activity in the mPFC

LPS significantly increased 5-HIAA levels in both the NAc and mPFC, without changing extracellular 5-HT levels. Previously, it was observed that LPS increases extracellular 5-HIAA levels in rats as well (Borowski et al., 1998; Connor et al., 1999; Merali et al., 1997). In the present study, DOV 216,303 pretreatment prevented the LPS-induced 5-HIAA formation in the mPFC (AUC values), suggesting that LPS increases SERT activity in this brain area. Remarkably, this suppression was less severe in the NAc than in the mPFC. Possibly, the dose of DOV 216,303 (5 mg/kg) was not high enough to completely inhibit SERT activity in the NAc (Caldarone et al., 2010). The stronger inhibitory effect of DOV 216,303 on SERT activity in the mPFC could also be explained by the fact that the mPFC is highly innervated by serotonergic neurons arising from the raphe nucleus, whereas the NAc is not (Molliver, 1987). This LPS-induced increase in SERT function is in correspondence with *in vitro*, *ex vivo* and *in vivo* studies, showing that LPS increases proinflammatory cytokines and thereby SERT function (within one hour) (Mossner et al., 1998; Zhu et al., 2006; Zhu et al., 2010). It has been shown that this process is p38 MAPK dependent (Zhu et al., 2004; Zhu et al., 2005; Zhu et al., 2010). Furthermore, there are strong indications that LPS- and proinflammatory cytokine-induced increases in SERT function are necessary for the development of both depression-like behavior and anhedonia. First of all, previously it was shown that LPS-induced depression-like behavior, as measured in the tail-suspension test (Zhu et al., 2010) and LPS-induced anhedonia, as measured in the ICSS paradigm (van Heesch et al., 2013a), was abolished in SERT knockout animals. Secondly, it has been demonstrated that prophylactic administration of SSRIs can be successfully used in a subgroup of patients, who are at risk of developing major depression during IFN-alpha anti-cancer treatment (Galvao-de Almeida et al., 2010). Furthermore, the SSRI fluoxetine decreases TNF-alpha-induced immobility time in the forced swim test (Kaster et al., 2012). Thus, altogether, this supports the hypothesis that LPS and/or proinflammatory cytokines play an important role in increasing SERT activity.

Effects of LPS on monoamine levels

Remarkably, LPS did not affect 5-HT levels in the NAc and mPFC, suggesting that depletion of 5-HT itself, is not the cause of anhedonia per se. This is supported by a meta-analysis study, showing that acute tryptophan depletion in humans leading to depletion of 5-H, cannot induce major depression in healthy subjects (Ruhe et al., 2007).

Previously, it was shown that peripheral LPS increases extracellular DA levels in the NAc of rats (Borowski et al., 1998). We only found a trend towards increased DA levels in the NAc of LPS-exposed mice. This is supported by the findings that LPS, TNF-alpha and IL-1 did not significantly affect extracellular DA levels in the NAc (Merali et al., 1997; van Heesch et al.2013b; van Heesch et al. unpublished results). In contrast to the NAc, LPS significantly increased DA levels in the mPFC. This increase in prefrontal DA is in agreement with a microdialysis study performed in LPS-treated rats (Dunn, 2006) and is probably caused by a very low density of DAT in the PFC (Sesack et al., 1998), even though DA has a high affinity for NET as well (Gresch et al., 1995; Gu et al., 1994).

Conclusions

In conclusion, here it is shown that peripherally injected LPS, that has been shown to induce anhedonia in mice, increases degradation of central monoamines in the NAc and mPFC. Since pretreatment with the triple reuptake inhibitor DOV 216,303 prevented LPS-induced monoamine metabolite formation we hypothesize that increased monoamine transporter activity is responsible for these increases in central monoamine degradation. To be more precise, the present findings suggest that peripheral LPS increases DAT activity in the NAc and increases both NET and SERT activity in the mPFC. However, more studies are needed to directly investigate via which molecular mechanisms SERT, DAT and NET activity in the NAc and mPFC can be increased by LPS and whether and how this is related to anhedonia.

5

Lipopolysaccharide-induced anhedonia is abolished in male serotonin transporter knockout rats: An intracranial self-stimulation study

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Abstract

A growing body of evidence suggests that proinflammatory cytokines contribute to the pathogenesis of depression. Previously, it has been shown that cytokines (e.g. interferon-α therapy) induce major depression in humans. In addition, administration of the cytokine-inducer lipopolysaccharide (LPS) provokes anhedonia (i.e. the inability to experience pleasure) in rodents. Furthermore, serum proinflammatory cytokine levels are increased in depressed patients. Nevertheless, the etiology of cytokine-induced depression is largely unknown. Previously, it has been shown that selective serotonin re-uptake inhibitors decrease serum proinflammatory cytokine levels and that proinflammatory cytokines increase activity of the serotonin transporter (SERT). The purpose of this study was to explore the effect of partial and complete lack of the SERT in LPS-induced anhedonia assessed in the intracranial self-stimulation (ICSS) paradigm. A single intraperitoneal injection of LPS was used to induce a proinflammatory immune response in male serotonin transporter wild type ($SERT^{+/+}$), heterozygous ($SERT^{+/-}$) and knockout ($SERT^{-/-}$) rats. Body weight and ICSS thresholds were measured daily. Although LPS reduced body weight in all genotypes, loss of body weight was less pronounced in $SERT^{-/-}$ compared to $SERT^{+/+}$ rats. Remarkably, LPS-induced anhedonia was totally abolished in $SERT^{-/-}$ rats and as expected was still present in $SERT^{+/+}$ and to a lesser extent in $SERT^{+/-}$ rats. Therefore, it is concluded that an intact SERT function is needed for proinflammatory cytokine-induced anhedonia and weight loss in rats.

Introduction

Major depressive disorder (MDD) is a complex disorder affecting 121 million people worldwide. Although, according to the World Health Organization depression is among the leading causes of disability, the exact etiology is largely unknown. Since the mid-seventies there is a growing body of evidence that reciprocal pathways between immune, endocrine and central nervous system are not only involved in physical health, but also in mental health. In this respect, there is an increasing interest in the putative involvement of the immune system in depression, especially in depression due to a general medical condition (Dantzer et al., 2008; Dantzer, 2009; Konsman et al., 2002). This hypothesis is supported by the following findings: first of all, it was shown that the cytokines IFN-α and IL-2, used effectively as therapy for hepatitis C and cancer, increase the risk for the development of depression (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987). Secondly, although the possibility that cytokines are also related to major depression in the absence of a medical condition has been debated, a recent meta-analysis concludes that the proinflammatory cytokines TNF-α and IL-6 are increased in serum of depressed patients (Dowlati et al., 2010). Furthermore, it has been demonstrated that lipopolysaccharide (LPS), a component of the outer membrane of gram negative bacteria that binds to toll-like receptor 4 (TRL4) leading to the rapid systemic release of proinflammatory cytokines, induces anhedonia in rats and mice as shown by increased thresholds in an intracranial self-stimulation (ICSS) paradigm (Borowski et al., 1998; Prins et al., 2011; van Heesch et al., 2012). Anhedonia is a core symptom of MDD and characterized by the inability to experience pleasure probably caused by reduced ability to experience reward.

Selective serotonin reuptake inhibitors (SSRIs) are the most prescribed antidepressants worldwide. Therefore the role of serotonin (5-HT) and the serotonin transporter (SERT) have been studied intensively in depression and an important role for altered serotonergic neurotransmission in MDD has been proposed (Albert et al., 2012). The SERT regulates 5-HT availability in the synaptic cleft and is therefore important in 5-HT driven processes. It was shown that inhibition of SERT by SSRIs suppresses proinflammatory cytokine production by T helper 1 (T_{H1}) cells in whole blood ex vivo (Diamond et al., 2006; Taler et al., 2007) and decreases serum proinflammatory cytokine levels in depressed patients (Hannestad et al., 2011a; Lee and Kim, 2006; Sutcigil et al., 2007). On the other hand, LPS and proinflammatory cytokines, e.g. TNF-α, which induce anhedonia (Borowski et al., 1998; Prins et al., 2011; van Heesch et al., 2012), increase SERT function (Mossner et al., 1998; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010). This suggests that people and animals with impaired SERT functioning, through pharmacologic intervention with SSRIs or genetic variations, respond less to an immune challenge. Previously, it was found that SSRIs reduced severity of IFN-α therapy-induced depressive symptoms (Capuron et al., 2002; Kraus et al., 2002; McNutt et al., 2012; Raison et al., 2007). In contrast, people with

decreased function of the SERT, caused by a short promoter version (SS) of the SERT gene, seem to be more vulnerable to become depressed during IFN-α therapy (Bull et al., 2009; Lotrich et al., 2009), although this could not always be replicated (Kraus et al., 2007). Therefore, more investigation is needed to determine the role of SERT in proinflammatory cytokine-induced depression.

SERT knock-out ($SERT^{-/-}$) rats show complete absence of the SERT, whereas rats heterozygous ($SERT^{+/-}$) for the SERT show 48-80% SERT activity compared to wild type animals ($SERT^{+/+}$) (Homberg et al., 2007). This makes the SERT knock-out rat a good subject to study the effects of partial or complete absence of the SERT on proinflammatory cytokine-induced anhedonia. Therefore, $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats were trained in the ICSS paradigm in order to investigate the role of the SERT in proinflammatory cytokine-induced anhedonia.

Methods

Animals

Male serotonin transporter knockout rats ($Slc6a4^{1Hubr}$), generated by ENU-induced mutagenesis (Smits et al., 2006), were generated, bred and reared in the animal facilities of the University of Utrecht. Animals were bred by crossing serotonin transporter heterozygous rats ($SERT^{+/-}$) in a quiet room. At the age of 21 days, pups were weaned and ear cuts were taken for genotyping. Animals were placed on a 12 h light-dark cycle with lights on at 6:00 am and off at 6:00 pm. Food and water were available *ad libitum*. For the experiment, 8 animals per genotype were selected on the base of age and body weight (280 – 350g), to prevent wrong electrode placements during surgery due to too low or too high body weight. Experimental animals were housed 4 per cage. Each animal was housed with littermates having the same genotype ($SERT^{+/+}$,$SERT^{+/-}$ or $SERT^{-/-}$) and undergoing the same treatment (saline or LPS). The study was carried out in accordance with the governmental guidelines and approved by the Ethical Committee for Animal Research of Utrecht University, The Netherlands.

Drugs

Escherichia Coli derived lipopolysaccharide (LPS) (Sigma, 0127:B8) was dissolved in 0.9% saline and prepared freshly on test days from the stock solution (0.5 mg/ml dissolved in 0.9% saline). 250 µg/kg LPS was administered intraperitoneally (i.p.) in a volume of 2 ml/kg. Control animals received injections of 0.9% saline in a volume of 2 ml/kg.

Body weight

Body weight of rats was measured daily around noon ($SERT^{+/+}$: n=8; $SERT^{+/-}$: n=8 and $SERT^{-/-}$: n=8). Body weight measured during the three days before saline or LPS injection was averaged and used to calculate body weight at baseline and assess delta body weight values at different time points after the saline or LPS injection.

Intracranial self-stimulation (ICSS)

ICSS surgery

Twenty-four rats ($SERT^{+/+}$: n=8; $SERT^{+/-}$: n=8 and $SERT^{-/-}$: n=8) were anesthetized by inhalation of a mixture of isoflurane gas (2%) and oxygen and placed in a stereotaxic instrument (Kopf, David Kopf Instruments). Lidocaine hydrochloride (2%) + adrenaline (0.001%) were applied in the incision as a local anesthetic. Twisted bipolar electrodes (cut to 11 mm) were implanted in the medial forebrain bundle. The coordinates were anteroposterior –0.5 mm; mediolateral +/– 1.7 mm from bregma; dorsoventral –8.3 mm from dura. The stereotaxic incisor bar was adjusted to 5 mm above the interaural line (Pellegrino et al., 1979). Electrodes were anchored with four screws and dental acrylic on the skull. All animals received the non-steroidal anti-inflammatory drug (NSAID) Rimadyl for pain relief (5 mg/kg, subcutaneously, twice a day, 4 injections in total). Animals were given one week to recover from surgery before start of the ICSS training.

ICSS experiment

One week after surgery animals were trained daily in the ICSS paradigm according to the discrete-trial current-intensity procedure as described before by Kornetsky and Esposito (Kornetsky and Esposito, 1979), Markou and Koob (Markou and Koob, 1992), Kenny and Markou (2006), Kenny et al. (Kenny et al., 2003; Kenny and Markou, 2006), Cryan et al. (Cryan et al., 2003) and Prins et al. (Prins et al., 2012). Each session consisted of four alternating series of descending and ascending current intensities starting with a descending series. A set of five trials was presented for each current intensity and the intensity was altered in 5 µA steps between blocks of trials. The initial current intensity was set approximately 40 µA above baseline thresholds so that the initial current intensity of each subject was within 100 to 300 µA. One-quarter turn of the wheel manipulandum within 7.5 sec after the presentation of a non-contingent electrical stimulation resulted in another electrical stimulus. The time to respond to the non-contingent electrical stimulus was recorded (response latency). After a positive or negative response (no turn of the wheel within 7.5 sec after the non-contingent stimulus) the inter-trial interval started with an

average duration of 10 sec (7.5-12.5 sec range) followed by another trial with the delivery of a non-contingent stimulus. The onset of the next trial was delayed in case rats responded during the inter-trial interval. Rats that were unable to learn the ICSS program during the training sessions were excluded from the ICSS experiment ($SERT^{+/-}$: n=3 and $SERT^{-/-}$: n=3), but not from the body weight determinations. After the establishment of steady ICSS thresholds (3 consecutive days during which ICSS thresholds varied by <10%), rats received an i.p. injection of either saline or LPS and were tested in the ICSS paradigm 1h, 4h, 24h, 48h, 72h, 96h, 120h, 144h and 360h after injection. Three weeks after the first injection, the experiment was repeated, as animals were tested in a cross-over design in such a way that all the animals received a saline and LPS injection ($SERT^{+/+}$: n=8; $SERT^{+/-}$: n=5; $SERT^{-/-}$: n=5). During each measurement ICSS thresholds and response latencies were recorded. ICSS thresholds measured during the three days before saline or LPS injection were averaged and used to calculate ICSS thresholds at baseline and delta ICSS thresholds at different time points after a saline or LPS injection. Increased ICSS thresholds compared to baseline measurements implies that activity of the reward circuitry is decreased (Markou and Koob, 1992). Response latencies obtained during the three baseline measurements were used to calculate percentage change from baseline values at each time point tested after the injections. After the last measurement, animals were decapitated and brains were removed for further analysis of electrode localization.

Probe placement

Dissected brains were quickly stored in formaldehyde until verification of electrode localization. The brains were placed in 30% sucrose solution and frozen two days later. Probe placements were verified on 60 µm cresyl violet stained sections obtained on a cryostat (Leica CM3050).

Statistical analysis

Body weight during surgery, baseline body weight, baseline ICSS thresholds and baseline ICSS response latencies were analyzed with use of one-way ANOVA, to detect differences in baseline between the different genotypes. When the variances across genotypes were not equal, data was analyzed with use of Welch's ANOVA. In case of a significant difference the Tukey or Games-Howell post hoc test was performed dependent on the test that was used (one-way ANOVA or Welch's, respectively).

Body weight data and ICSS current-threshold data are expressed as the difference from baseline data (delta body weight and delta ICSS thresholds, respectively). Response latency data are expressed as a percentage of the baseline data. Data of saline-treated animals were analyzed by repeated measures analysis of variance (repeated measures ANOVA) with time

(9 levels: 0 h, 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 360 h (delta body weight) or 10 levels: 0 h, 1 h, 4 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 360 h (delta ICSS thresholds and response latencies)) as within subject factor and genotype ($SERT^{+/+}$. $SERT^{+/-}$ and $SERT^{-/-}$) as between subject factor. In case of no significant time x genotype interaction or genotype effect, saline-$SERT^{+/+}$, saline-$SERT^{+/-}$ and saline-$SERT^{-/-}$ animals were grouped in the vehicle-control group. Then, delta body weight, delta ICSS thresholds and response latencies were analyzed by repeated measures ANOVA with time (9 levels for delta body weight as shown above or 10 levels for delta ICSS thresholds and response latencies as shown above) as within subject factor and group (vehicle-control, LPS-$SERT^{+/+}$, LPS-$SERT^{+/-}$ or LPS-$SERT^{-/-}$) as between subject factor. In case of a significant time x group interaction one-way ANOVA's were performed for each time point after injection. In case of a significant difference, data of a specific time point was analyzed with the Dunnett's post hoc test. Vehicle-control was used as the control category. When the assumption of sphericity was violated, reported results were corrected by the Greenhouse-Geisser procedure.

Area under the curve (AUC) values of delta body weight, ICSS thresholds and ICSS response latencies were analyzed with use of one-way ANOVA. When there was a significant difference between groups, a Dunnett's post hoc test was performed with vehicle-controls as the control category.

All data were analyzed using the SPSS 20 software statistical package.

Results

Body weight

Mean body weights (± Standard Error of the Mean (S.E.M.)) during surgery were 325.4 ± 8.1 g, 304.0 ± 6.3 g and 312.4 ± 11.1 g in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively. At this point there were no significant differences in body weight between genotypes. Several weeks later, at start of injection, body weight varied according genotype. Mean baseline body weights (± S.E.M.) measured three days before saline or LPS injections were 520.2 ± 8.2 g, 469.7 ± 8.1 g and 443.6 ± 8.8 g in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively ($F(2,47) = 21.7$, $p < 0.001$). Post hoc tests showed that $SERT^{+/+}$ rats had significantly higher body weights compared to $SERT^{+/-}$ and $SERT^{-/-}$ animals. Repeated measures ANOVA revealed that there were no differences in delta body weight of saline-$SERT^{+/+}$, saline-$SERT^{+/-}$ and saline-$SERT^{-/-}$ rats, since there was neither time x treatment interaction nor an effect of treatment. After merging the saline-treated animals together in the vehicle-control group, repeated measures ANOVA revealed a significant time x group

interaction ($F(8.7,127.2) = 26.7$, $p < 0.001$, $\varepsilon = 0.361$) and significant effect of group ($F(3,44) = 67.4$, $p < 0.001$). One-way ANOVA's per time point showed significant differences at each time point after the saline or LPS injection. LPS-SERT$^{+/+}$ rats already showed a significant decrease in body weight 1 h after exposure to LPS ($p < 0.05$). At time points 24 h until 144 h after exposure to LPS, all genotypes treated with LPS showed a significant decrease in body weight compared to the vehicle-control group ($p < 0.001$) (***Figure 5.1: A***). At time point 360 h after exposure to LPS the difference compared to vehicle-controls was the largest in LPS-SERT$^{+/+}$ rats ($p < 0.001$), followed by LPS-SERT$^{+/-}$ ($p < 0.01$) and LPS-SERT$^{-/-}$ rats ($p < 0.05$). One-way ANOVA of corresponding AUC values revealed a significant difference between the 4 groups ($F(3,13.1) = 100.0$, $p < 0.001$) (***Figure 5.1: B***). Post hoc analysis showed that all three genotypes treated with LPS differed significantly from the vehicle-control group ($p < 0.001$).

Intracranial self-stimulation

Probe placement

Histology revealed that the electrodes of all animals that were used in the present study (SERT$^{+/+}$: n=8; SERT$^{+/-}$: n=5; SERT$^{-/-}$: n=5) were localized in the medial forebrain bundle. In the rats that were unable to learn the ICSS program during the training sessions and were excluded from ICSS experiment (SERT$^{+/-}$: n=3 and SERT$^{-/-}$: n=3) misplacement of electrodes was found.

Intracranial self-stimulation thresholds

Mean baseline ICSS thresholds (± S.E.M.) were 120.4 ± 14.2 µA, 95.8 ± 8.5 µA and 172.7 ± 23.0 µA in SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats, respectively, and significantly differed between genotypes ($F(2,18.7) = 5.2$, $p < 0.05$). Post hoc analysis revealed significant higher ICSS baseline thresholds in SERT$^{-/-}$ compared to SERT$^{+/-}$ rats ($p < 0.05$). Delta ICSS thresholds of SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats exposed to saline did not differ, since repeated measures ANOVA revealed no time x treatment interaction, nor an effect of treatment. After merging the saline-treated animals together, repeated measures ANOVA of delta ICSS thresholds showed a significant time x group interaction and significant effect of group ($F(10.2,108.9) = 4.7$, $p < 0.001$, $\varepsilon = 0.378$ and $F(3,32) = 3.9$, $p < 0.05$, respectively) (***Figure 5.2: A***). Compared to the vehicle-control group, ICSS thresholds were significantly elevated in the LPS-SERT$^{+/+}$ and LPS-SERT$^{+/-}$ group at 1, 4, 24 h and 1 h after exposure to LPS, respectively. Delta ICSS thresholds of SERT$^{-/-}$ rats did not differ from the vehicle-control group at any time point after the LPS injection. One-way ANOVA

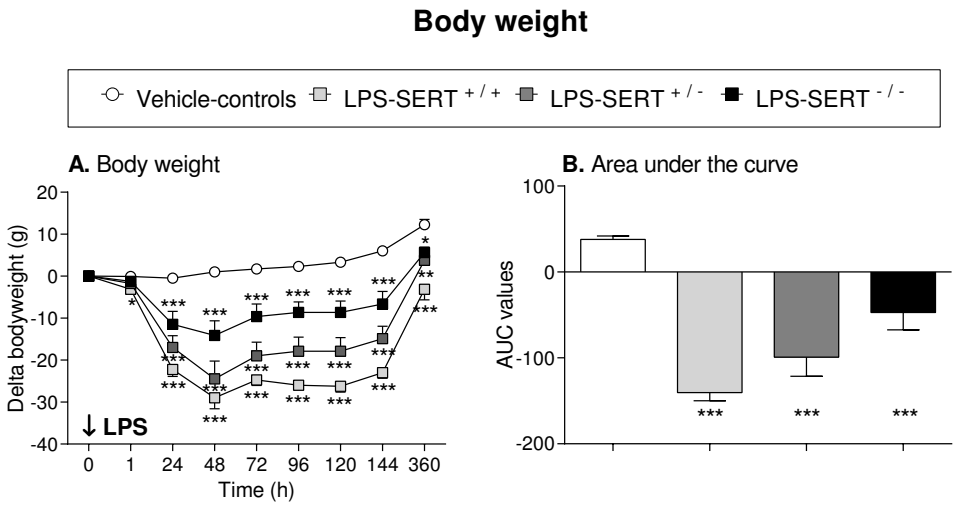

Figure 5.1: A: The effect of i.p. injected LPS on delta body weight and **B:** corresponding area under the curve values in $SERT^{+/+}$ (light grey, n=8), $SERT^{+/-}$ (dark grey, n=8) and $SERT^{-/-}$ rats (black, n=8) compared to vehicle-controls (white, n=24). The vehicle-control group consists of $SERT^{+/+}$ (n=8), $SERT^{+/-}$ (n=8) and $SERT^{-/-}$ rats (n=8) exposed to saline. Body weight was measured after exposure to vehicle or LPS. Delta body weight and AUC values are presented as mean ± standard error of the mean; statistically significant results are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle-controls.

of ICSS threshold AUC values revealed that there was a significant difference between the four groups ($F(3,35) = 4.1$, $p < 0.05$) (***Figure 5.2: B***). Post hoc analysis showed that only $SERT^{+/+}$ rats had significantly higher AUC values compared to the vehicle-control group ($p < 0.01$).

Response latencies

Mean baseline response latencies (± S.E.M.) were 2.81 ± 0.21 s, 2.78 ± 0.08 s and 3.34 ± 0.26 s in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively, and did not significantly differ. Repeated measures ANOVA of percentage change from baseline response latencies of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats exposed to saline revealed no time x treatment interaction, nor an effect of treatment. After the saline-treated animals were merged in the vehicle-control group, repeated measures ANOVA revealed a significant time x group interaction and a trend towards a group effect ($F(13.0,138.8) = 2.2$, $p < 0.05$, $\varepsilon = 0.480$ and

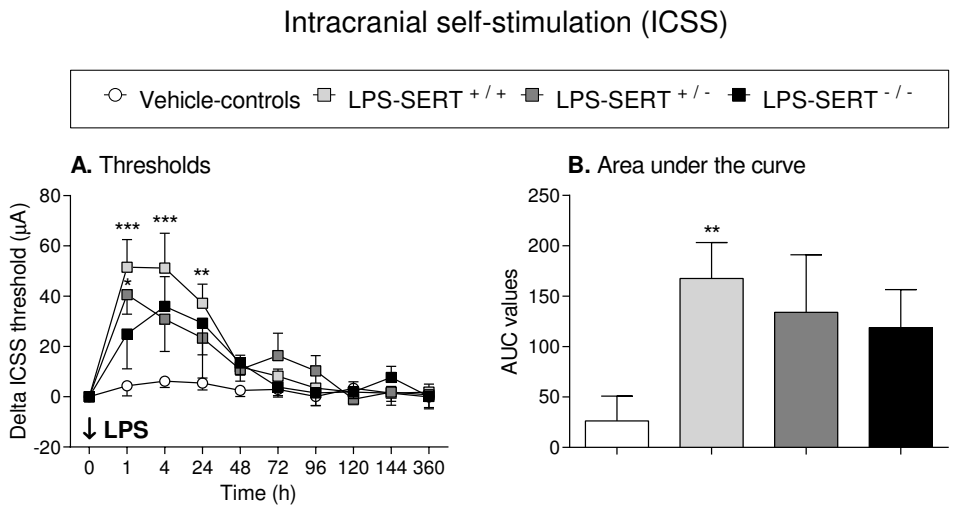

Figure 5.2: A: The effect of i.p. injected LPS on delta ICSS thresholds and **B:** corresponding area under the curve values in SERT$^{+/+}$ (light grey, n=8), SERT$^{+/-}$ (dark grey, n=5) and SERT$^{-/-}$ rats (black, n=5) compared to vehicle-controls (white, n=18). The vehicle-control group consists of SERT$^{+/+}$ (n=8), SERT$^{+/-}$ (n=5) and SERT$^{-/-}$ rats (n=5) exposed to saline. ICSS thresholds were measured after exposure to vehicle or LPS. Delta ICSS thresholds and AUC values are presented as mean ± standard error of the mean; statistically significant results are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle-controls.

$F(3,32) = 2.4$, $p = 0.083$, respectively) (***Figure 5.3: A***). One-way ANOVA per time point revealed that response latencies differed significantly between the groups at 4 h after saline or LPS injection ($F(3,35) = 4.8$, $p < 0.01$). At this time point LPS-SERT$^{+/+}$ rats had significantly lower response latencies compared to vehicle-controls ($p < 0.05$),while response latencies of LPS-SERT$^{+/-}$ and LPS-SERT$^{-/-}$ rats did not differ from the vehicle-control group. For area under the curve data of response latencies there was only a trend towards a difference between the four groups ($F(3,35) = 2.4$, $p = 0.085$ (***Figure 5.3: B***).

Discussion

To our knowledge we are the first to show that lipopolysaccharide (LPS)-induced anhedonia is abolished in SERT$^{-/-}$ rats when compared to SERT$^{+/+}$ and SERT$^{+/-}$ animals, which still respond to a peripheral LPS challenge with a significant increase in intracranial

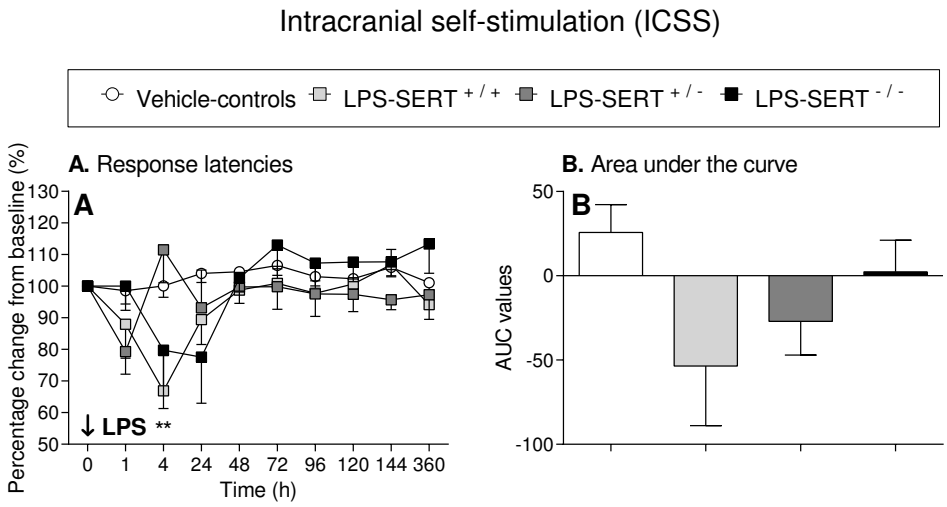

Figure 5.3: A: The effect of i.p. injected LPS on ICSS response latencies and **B:** corresponding area under the curve values in SERT$^{+/+}$ (light grey, n=8), SERT$^{+/-}$ (dark grey, n=5) and SERT$^{-/-}$ rats (black, n=5) compared to vehicle-controls (white, n=18). The vehicle-control group consists of SERT$^{+/+}$ (n=8), SERT$^{+/-}$ (n=5) and SERT$^{-/-}$ rats (n=5) exposed to saline. ICSS response latencies were measured after exposure to vehicle or LPS. ICSS response latencies and AUC values are presented as mean ± standard error of the mean; statistically significant results are indicated as ** $p < 0.01$ compared to vehicle-controls.

self-stimulation (ICSS) thresholds, reflecting the inability to experience pleasure (anhedonia). In addition, LPS produced less weight loss in SERT$^{-/-}$ rats than in SERT$^{+/+}$ and SERT$^{+/-}$ rats. In agreement, LPS enhanced immobility in the tail suspension test in SERT$^{+/+}$ mice, but failed to do so in SERT$^{-/-}$ mice (Zhu et al., 2010). These data indicate that LPS-induced depressive-like behavior is also not observed in SERT$^{-/-}$ mice.

It is well known that peripheral LPS administration induces anhedonia in several animal tests. Previously, it has been reported that LPS lowers the consumption and preference for saccharin and sucrose (Frenois et al., 2007; Larson, 2006; Yirmiya, 1996) and suppresses male sexual behavior (Yirmiya, 1996). Furthermore, it has been shown that LPS increases ICSS thresholds in rats and mice (Borowski et al., 1998; Prins et al., 2011; van Heesch et al., 2012). We show that response latencies did not differ between genotypes at baseline. Furthermore, response latencies did not increase after the LPS injection. Actually, we show that response latencies decreased in SERT$^{+/+}$ due to LPS. Thus, it is likely that the observed increases in ICSS thresholds are not due to decreased motor performance in the ICSS test (Stoker and Markou, 2011).

SERT function is probably involved in LPS-induced anhedonia, because proinflammatory cytokines increase SERT function both in vitro and in vivo (Mossner et al., 1998; Zhu et al., 2006; Zhu et al., 2010). TLR 4 activation by LPS is known to activate p38 MAPK (Bode et al., 2012) and it has been shown that SERT activity can be increased by stimulation of the p38 MAPK pathway (Zhu et al., 2004; Zhu et al., 2006; Zhu et al., 2010). In agreement, inhibition of this pathway decreases LPS-induced 5-HT uptake and abolishes LPS-induced increased immobility time in the tail suspension test (Zhu et al., 2004; Zhu et al., 2006; Zhu et al., 2010). Collectively, together with our finding that LPS-induced anhedonia is abolished in $SERT^{-/-}$ rats, but not in $SERT^{+/+}$ animals, it can be concluded that increased SERT activity due to inflammation may play an important role in the development of depression. This is in agreement with the finding that the SSRI, fluoxetine, decreases TNF-α-induced immobility time in the forced swim test (Kaster et al., 2012). Furthermore, there are indications that prophylactic administration of SSRIs can be successfully used in a subgroup of patients who are at risk of developing MDD during IFN-α treatment (Galvao-de Almeida et al., 2010). Interestingly, SERT expression and function is reduced in $SERT^{+/-}$ rats and therefore these rats are somewhat comparable to the human SS 5-HTT genotype. Therefore, it is not surprising that LPS challenge in $SERT^{+/-}$ rats resulted in an increase in ICSS thresholds and decrease in body weight that was (although not significant) between the values found in $SERT^{+/+}$ and $SERT^{-/-}$ rats.

In the present study, non-treated $SERT^{-/-}$ rats had significantly higher baseline ICSS thresholds when compared to non-treated $SERT^{+/-}$ animals, but did not differ from $SERT^{+/+}$ rats. It is unlikely that the LPS-induced changes in ICSS thresholds are due to differences in electrode placement between genotypes, because after histological examination it was shown that all electrodes were localized in the medial forebrain bundle. By showing difference from baseline data instead of a percentage of the baseline data, the chance that probe placements influenced the outcome of the study is further reduced. Interestingly, previously it has been reported that sucrose consumption was decreased and immobility time in the forced swim test was increased in non-treated $SERT^{-/-}$ rats as compared to non-treated $SERT^{+/+}$ animals (Homberg et al., 2010; Olivier et al., 2008b), suggesting that under normal conditions $SERT^{-/-}$ rats may already differ in anhedonia levels and behavioral measures of depression.

LPS affected body weight in all three genotypes, which can be explained by the fact that LPS-induced increases in cytokine levels reduce both appetite and body weight (Langhans and Hrupka, 1999; Langhans, 2007). AUC values of delta body weight suggest that this decrease was less severe in $SERT^{-/-}$ than in $SERT^{+/+}$ rats and that decrease in body weight of $SERT^{+/-}$ rats was in between the values obtained from $SERT^{+/+}$ and $SERT^{-/-}$ rats. It is well known that there is an inverse relationship between brain extracellular 5-HT concentrations and food intake (Hutson et al., 1988; Schwartz et al., 1989). Increased extracellular 5-HT levels have been found in the hippocampus, nucleus accumbens and

prefrontal cortex of $SERT^{-/-}$ when compared to $SERT^{+/+}$ rats (Homberg et al., 2007; Olivier et al., 2008b, van Heesch et al., unpublished data), which makes it plausible that 5-HT levels in the hypothalamus are also increased, probably explaining the lower body weight in $SERT^{-/-}$ rats.

In conclusion, peripheral administration of LPS induces anhedonia in $SERT^{+/+}$ and $SERT^{+/-}$ rats, probably via the release of cytokines that stimulate the p38 MAPK pathway and consequently increase SERT function. Since SERTs are not expressed in $SERT^{-/-}$ rats, LPS cannot affect SERT activity in these animals. Therefore, it is hypothesized that increased SERT function in humans with inflammatory diseases increases the risk in these patients to develop co-morbid major depression, especially anhedonia.

6

Lipopolysaccharide-increased brain serotonin metabolism is absent in male serotonin transporter knockout rats: An in vivo microdialysis and cytokine profile study

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Abstract

It is well known that bacterial lipopolysaccharides (LPS) induce anhedonia, i.e. the inability to experience pleasure. Previously, we have found that LPS-induced anhedonia is abolished in serotonin transporter knockout ($SERT^{-/-}$) rats. It is unknown, however, whether this insensitivity of $SERT^{-/-}$ rats is due to a decreased release of proinflammatory cytokines in response to LPS or the necessity of SERT activity to induce anhedonia. In the present study, the effect of an intraperitoneal LPS injection on extracellular concentrations of serotonin and its metabolite 5-HIAA was investigated by *in vivo* microdialysis in the nucleus accumbens (NAc) and medial prefrontal cortex of wild type ($SERT^{+/+}$), heterozygous ($SERT^{+/-}$) and $SERT^{-/-}$ rats. Furthermore, serum and forebrain cytokines were measured. Here we show that, in both the NAc and mPFC, LPS-induced 5-HIAA formation is significantly increased in $SERT^{+/+}$ rats, whereas this increase is partly or totally abolished in $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively. LPS is still able to normally increase serum and forebrain cytokines in $SERT^{-/-}$ rats. Therefore, the present study supports the hypothesis that increased SERT function plays an important role in LPS-induced anhedonia.

Introduction

An increasing amount of evidence suggests that there is an important role for proinflammatory cytokines in major depression, particularly in depression due to a general medical condition (Dantzer et al., 2008; Dantzer, 2009; Konsman et al., 2002; Pollak and Yirmiya, 2002). For example, patients with chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease or psoriasis, have an increased risk to become depressed (Akay et al., 2002; Hauser et al., 2011; Isik et al., 2007; Loftus et al., 2011). Furthermore, interferon-α (IFN-α) and interleukin-2 (IL-2), effectively used as therapy for hepatitis C and some cancers, increase the risk for the development of depression (Capuron et al., 2004; Heinze et al., 2010; Renault et al., 1987). Altogether this suggests that peripheral proinflammatory cytokines can modify brain functioning and increase the risk to develop depression.

Anhedonia i.e. the inability to experience pleasure and a core symptom of major depression, can be assessed in rodents with the intracranial self-stimulation (ICSS) procedure (Kenny et al., 2003). Peripherally injected bacterial lipopolysaccharide (LPS), a potent activator of the immune system leading to the rapid release of proinflammatory cytokines, has been shown to induce anhedonia in rats (Borowski et al., 1998; van Heesch et al., 2013a). Interestingly, LPS-induced anhedonia is not observed in serotonin transporter knock-out ($SERT^{-/-}$) rats, while LPS-induced anhedonia was decreased in rats heterozygous for the SERT ($SERT^{+/-}$) compared to wild type animals ($SERT^{+/+}$) (van Heesch et al., 2013a). Although the serotonin transporter (SERT) is completely absent in $SERT^{-/-}$ rats, $SERT^{+/-}$ rats have been shown to have 48-80% SERT activity compared to wild type animals ($SERT^{+/+}$) (Homberg et al., 2007). This suggests that absence of SERT protects the animals from LPS-induced anhedonia.

The exact reason why $SERT^{-/-}$ rats are not sensitive for LPS-induced anhedonia is still unknown. However, since SSRIs are supposed to alleviate depression by increasing 5-HT availability through inhibition of SERT, differences in 5-HT availability could be responsible for the resistance to LPS-induced anhedonia in $SERT^{-/-}$ rats. Furthermore, there are strong indications for an interaction between proinflammatory cytokines and SERT. For instance, it has been shown that the antidepressants SSRIs (selective serotonin reuptake inhibitors) suppress cytokine production, such as TNF-α, by T helper-1 (T_{H1}) cells in whole blood *ex vivo* (Diamond et al., 2006; Taler et al., 2007) and decrease TNF-α and IL-6 levels in depressed patients (Hannestad et al., 2011a). Furthermore it has been demonstrated that LPS and proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α), increase SERT function (Mossner et al., 1998; Ramamoorthy et al., 1995; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010). It is unknown, however, whether the absence of LPS-induced anhedonia in $SERT^{-/-}$ rats is due to decreased proinflammatory cytokine release in

response to LPS or the necessity of enhanced SERT activity to induce anhedonia. The aim of the present study was to get a better insight in the role of SERT in LPS-induced anhedonia. Therefore, we investigated how $SERT^{-/-}$ and $SERT^{+/-}$ rats respond to an LPS-induced immune challenge in respect to serotonergic neurotransmission in the brain and cytokine production in comparison to $SERT^{+/+}$ rats. To answer these questions, a microdialysis experiment was performed in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats to assess how a peripheral LPS challenge known to induce anhedonia (van Heesch et al., 2013a) affects serotonergic neurotransmission in each genotype. Furthermore, serum and forebrain cytokine levels were measured in each genotype 4 h after exposure to LPS.

Methods

Animals

Male serotonin transporter ($Slc6a4^{1Hubr}$) knockout rats generated by *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis (Smits et al., 2006) were bred and reared in the animal facilities of the University of Utrecht. Animals were bred by crossing serotonin transporter heterozygous rats ($SERT^{+/-}$) in a quiet room. At the age of 21 days, pups were weaned and ear cuts were taken for genotyping. Animals were placed on a 12 h light-dark cycle with lights on at 6:00 am and of at 6:00 pm. Food and water were available *ad libitum*. Animals were housed 4 per cage. Each animal was housed with littermates having the same genotype ($SERT^{+/+}$, $SERT^{+/-}$ or $SERT^{-/-}$) and undergoing the same treatment (saline or LPS). Experiments started once animals were weighing 290-350 g. The study was carried out in accordance with the Dutch governmental guidelines and approved by the Ethical Committee for Animal Research of Utrecht University, The Netherlands.

Drugs

Escherichia Coli derived lipopolysaccharide (LPS) (Sigma, 0127:B8) was dissolved in 0.9% saline and prepared freshly on test days from the stock solution (0.5 mg/ml dissolved in 0.9% saline). Two hundred fifty (250) µg/kg LPS was administered intraperitoneally (i.p.) in a volume of 2 ml/kg. Control animals received i.p. injections of 0.9% saline in a volume of 2 ml/kg.

Microdialysis

Microdialysis surgery

In total 48 rats were anesthetized by inhalation of a mixture of isoflurane gas (2%) and oxygen and placed in a stereotaxic instrument. Two cuprofane microdialysis probes per rat were implanted. One probe was implanted in the nucleus accumbens (NAc), the other in the medial prefrontal cortex (mPFC) (MAB 4.6.2 CU and MAB 4.7.3 CU, for NAc and mPFC, respectively (Microbiotech)). The coordinates of the NAc and mPFC were anterioposterior +1.6 mm; mediolateral +1.8 mm (under a 0^o angle) from bregma; dorsoventral –8.2 mm from skull surface and anterioposterior +3.2 mm; mediolateral +1.0 mm (under a 0^o angle) from bregma; dorsoventral –4.0 mm from skull surface, respectively (G. Paxinos, 2001). Probes were anchored with nonacrylic dental cement on the scull. After implantation of the microdialysis probes, rats were housed individually and placed in the microdialysis room until the end of the experiment.

Microdialysis experiment

The microdialysis experiment was performed in conscious freely moving rats, one day after implantation of the microdialysis probes. A pump (KdScientific Pump 220 series, USA) perfused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant flow rate of 0.02 ml/h. During microdialysis, the flow rate was set at 0.09 ml/h. At 8:00 am rats were connected to a dual channel swivel (type 375/D/22QM, Microbiotech) which allowed them to move freely. Three hours after connection 30-minute samples were manually collected in vials containing 15 µl of 0.1 M acetic acid and frozen at –80 ^oC until analysis with HPLC. From 11:00 am until 1:00 pm four baseline samples were collected. Subsequently, the animals were injected i.p. with saline ($SERT^{+/+}$ n=8; $SERT^{+/-}$ n=8 and $SERT^{-/-}$ n=8) or LPS ($SERT^{+/+}$ n=8; $SERT^{+/-}$ n=8 and $SERT^{-/-}$ n=8). During the whole experiment, 12 samples were collected in total. At the end of the microdialysis experiment all animals were sacrificed immediately. The brains were dissected and stored in formaldehyde to verify probe localization later on.

HPLC

Microdialysis samples were stored at –80 °C until analysis. Serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, The Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 x 50 mm, 3 µm

particle size) in combination with detector cell 1, separated and detected 5-HT, whereas column 2 (NeuroSep 115 C18 1 x 150 mm, 3 µm particle size) in combination with detector cell 2, separated and detected 5-HIAA. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 18.5 % Methanol and 400 mg/L OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5 % methanol and 700 mg/L OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. 5-HT and 5-HIAA were detected electrochemically using µVT-03 flow cells (Antec) with glassy carbon working electrodes. Potential settings were for 5-HT +0.30 V versus Ag/AgCl and for 5-HIAA +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec). The limit of detection was 0.05 nM (S/N ratio 3:1).

Histology

Two days before brain slicing, the brains were transferred from formaldehyde to a 30% sucrose solution. Probe placements were verified on 60 µm cresyl violet stained sections that were cut on a cryostat (Leica CM 3050). Data were discarded when the microdialysis probe was outside the NAc or mPFC.

Cytokine measurements

Serum and brain preparation

Forty-seven rats were injected with saline ($SERT^{+/+}$: n = 7, $SERT^{+/-}$: n = 9 or $SERT^{-/-}$: n = 7) or LPS ($SERT^{+/+}$: n = 8, $SERT^{+/-}$: n = 7 or $SERT^{-/-}$: n = 9). Four hours later, animals were anesthetized with i.p. sodium pentobarbital (60 mg/ml/animal). Once complete anesthesia was ensured, a blood sample was drawn from the left ventricle of the hart (500 µl). Blood samples were mixed with 28 µl 0.21 M EDTA and stored on ice. After blood withdrawal the animal was perfused with saline to clear all the blood from the rat. Subsequently the animal was decapitated and the brain was taken out. Then the brain was quickly frozen in dry ice cooled isopentane and placed on dry ice. At the end of all the procedures, blood samples were centrifuged for 10 min (Eppendorf centrifuge 5417R: 15000 r.p.m., 4 ^{o}C) and 0.2 ml serum was collected. Serum samples and brains were stored in –80^{o}C.

Collection of brain tissue

The forebrains were separated from the brains (6.70 mm – 3.70 mm from Bregma, Paxinos & Watson Brain atlas) at –10 ^{o}C and collected in 7 ml Precellys Lysing Kit vials. The forebrains were weighted and stored again in –80 ^{o}C until determination of protein content.

Protein determination

1 ml buffer (10 ml PBS + 1 cOmplete protease inhibitor cocktail tablet, Roche) was added to each 100 mg brain tissue. Then samples were homogenized in the Precellys 24 (Bertin Technologies) for 20 sec. Subsequently, samples were centrifuged (4 ^{o}C, 14000 rpm, 15 min, Eppendorf Centrifuge 5417R) and supernatant was collected. Protein determinations were done with a bicinchoninic acid (BCA) kit and measured in the iMark Microplate Reader (BioRad). Then samples were diluted to 2 mg protein per ml PBS and stored in –80^{o} until cytokine establishment in the multiplex assays for quantification.

Multiplex assay for quantification of cytokine

Serum cytokines were measured with use of the Rat Cytokine 10-Plex Panel kit (Invitrogen, Catalog#: LRC0002, Lot#: 820265) and a Luminex 200 (Luminex Corporation). Prior to the assay, serum samples were diluted fivefold and tenfold in assay diluent (Invitrogen). Each sample, serum samples as well as forebrain homogenates (2 mg protein/ml), was assayed for rat granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40/p70) and TNF-α. Seven concentrations were used for a standard curve. Standard 1 was made by diluting the reconstituted standard stock (1 ml assay diluent added to the provided stock (Invitrogen, Rt Cytokine 10-Plex Standard, Part#: SM0562, Lot: 491428)) 3 times in assay diluent. Subsequently threefold serial dilutions were made in assay diluent and used to determine a standard curve. The concentration ranges of the standards were: GM-CSF: 1773.3-2.4 pg/ml, IFN-γ: 1450.0-2.0 pg/ml, IL-1α: 10553.3-14.5 pg/ml, IL-1β: 5613.3-7.7 pg/ml, IL-2: 11340.0-15.6 pg/ml, IL-4: 680.0-0.9 pg/ml, IL-6: 2766,7-3.8 pg/ml, IL-10: 12100.0-16.6 pg/ml, IL-12 (p40/p70): 3463.3-4.8 pg/ml and TNF-α: 420.0-0.6 pg/ml. After completion of all steps in the assay, the plates were read in the Luminex® 200. Serum and brain homogenates of each animal were run in duplicate, and the resultant determinations were averaged. In some cases, the concentrations of cytokines were below detection thresholds. In these cases the lowest detected concentration was substituted. For brain samples (undiluted), the lowest measured standard curve value functioned as a cut-off point (GM-CSF: 7.30 pg/ml, IFN-γ: 5.97 pg/ml, IL-1α: 14.48 pg/ml, IL-1β: 23.10 pg/ml, IL-2: 420.00 pg/ml, IL-4: 0.93 pg/ml, IL-6: 11.39 pg/ml, IL-10: 49.79 pg/ml, IL-12 (p40/p70): 42.76 pg/ml and TNF-α: 5.19 pg/ml). The cut-off point for serum samples was 7.5 times higher,

because these samples were diluted 5 and 10 times (mean 7.5 times) (GM-CSF: 54.73 pg/ml, IFN-γ: 44.75 pg/ml, IL-1α: 108.57 pg/ml, IL-1β: 173.25 pg/ml, IL-2: 3150.00 pg/ml, IL-4: 7.00 pg/ml, IL-6: 85.39 pg/ml, IL-10: 373.46 pg/ml, IL-12 (p40/p70): 320.68 pg/ml and TNF-α: 38.89 pg/ml). Values below cut-off points were substituted by half of the cut-off point value. This substitution shifted values closer to the overall mean, decreasing the likelihood of a type I error.

Statistics

Mean NAc and mPFC baseline 5-HT and 5-HIAA levels in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats were analyzed with use of one-way ANOVA. Due to basal differences in 5-HT and 5-HIAA levels between genotypes, we decided to present microdialysis data as a percentage of the baseline data for each genotype separately. Then, for each genotype, measurements were expressed as a percentage of baseline and analyzed by repeated measures ANOVA with time (4 levels: –90 min, –60 min, –30 min and 0 min) as within subject factor and treatment (saline or LPS) as between subject factor to exclude differences between the saline and LPS groups at baseline. Subsequently, post-injection data was compared in a repeated measures ANOVA with time (8 levels: 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min and 240 min) as within subject factor and treatment (saline or LPS) as between subject factor. In case of a significant time x treatment interaction, effects of LPS on individual time points were analyzed with post hoc *t*-tests with treatment (saline or LPS) as the grouping variable. When the assumption of sphericity was violated, the results were corrected by the Greenhouse-Geisser procedure.

Serum and forebrain cytokine levels were analyzed with two-way ANOVAs with genotype and treatment as fixed factors. In case of a significant treatment effect, post hoc *t*-tests for the effect of treatment (saline or LPS) per genotype were performed. All data were analyzed using the SPSS 20 software statistical package.

Results

Microdialysis

Histology

Due to a bad badge of 2 mm membrane length microdialysis probes, we were unable to collect microdialysis samples of 7 animals. In all these animals the microdialysis probes

were obstructed. Furthermore, we had to exclude another 8 animals, due to wrong probe placement. So, in total, data of 15 rats was lost and as a consequence the groups for the microdialysis study in the NAc were small. The $SERT^{+/+}$-saline, $SERT^{+/+}$-LPS, $SERT^{+/-}$-saline, $SERT^{+/-}$-LPS, $SERT^{-/-}$-saline and $SERT^{-/-}$-LPS group consisted of 6, 7, 3, 7, 5 and 5 animals, respectively.

The dropout rates in the mPFC were smaller. For this brain area 4 animals had to be excluded from data analysis. In one animal the microdialysis probe was obstructed. In 2 other rats the outlet of the microdialysis probe was broken due to scratching and grooming during the night and therefore it was not possible to connect these animals to the microdialysis system. Another rat had to be excluded from data analysis due to wrong probe placement. Because of these drop outs the $SERT^{+/+}$-saline, $SERT^{+/+}$-LPS, $SERT^{+/-}$-saline, $SERT^{+/-}$-LPS, $SERT^{-/-}$-saline and $SERT^{-/-}$-LPS group existed of 7, 7, 8, 7, 8 and 7 rats, respectively.

Baseline 5-HT and 5-HIAA levels in the NAc and mPFC of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats

Under basal conditions 5-HT levels in the NAc as well as in the mPFC were significantly higher in $SERT^{-/-}$ rats compared to $SERT^{+/+}$ (NAc: $p < 0.001$ and mPFC: $p < 0.001$) and $SERT^{+/-}$ rats (NAc: $p < 0.001$ and mPFC: $p < 0.001$) (NAc: $F(2,18.7) = 56.5$, $p < 0.001$ and mPFC: $F(2,43) = 52.3$, $p < 0.001$) (***Figure 6.1: A*** and ***Figure 6.2: A*** for the NAc and mPFC, respectively). Remarkably, $SERT^{+/+}$ and $SERT^{+/-}$ rats did not differ from each other in respect to extracellular 5-HT levels in both brain areas. Also extracellular 5-HIAA levels differed significantly between genotypes in both brain structures (NAc: $F(2,33) = 21.1$, $p < 0.001$ and mPFC: $F(2,43) = 36.0$, $p < 0.001$) (***Figure 6.1: E*** and ***Figure 6.2: E*** for the NAc and mPFC, respectively). In the NAc as well as in the mPFC $SERT^{-/-}$ rats had significantly lower extracellular 5-HIAA levels compared to $SERT^{+/+}$ (NAc: $p < 0.001$ and mPFC: $p < 0.001$) and $SERT^{+/-}$ rats (NAc: $p = 0.008$ and mPFC: $p < 0.001$). Furthermore, extracellular 5-HIAA levels of $SERT^{+/-}$ rats were significantly lower than 5-HIAA levels of $SERT^{+/+}$ rats in both brain areas (NAc: $p = 0.016$ and mPFC: $p = 0.001$).

Repeated measures ANOVA for the first four baseline measurements revealed neither a signifcant time x treatment interaction nor a significant effect of treatment for 5-HT and 5-HIAA in the NAc as well as in the mPFC (***Figure 6.1*** and ***Figure 6.2***). Thus, before exposure to saline or LPS, groups were comparable.

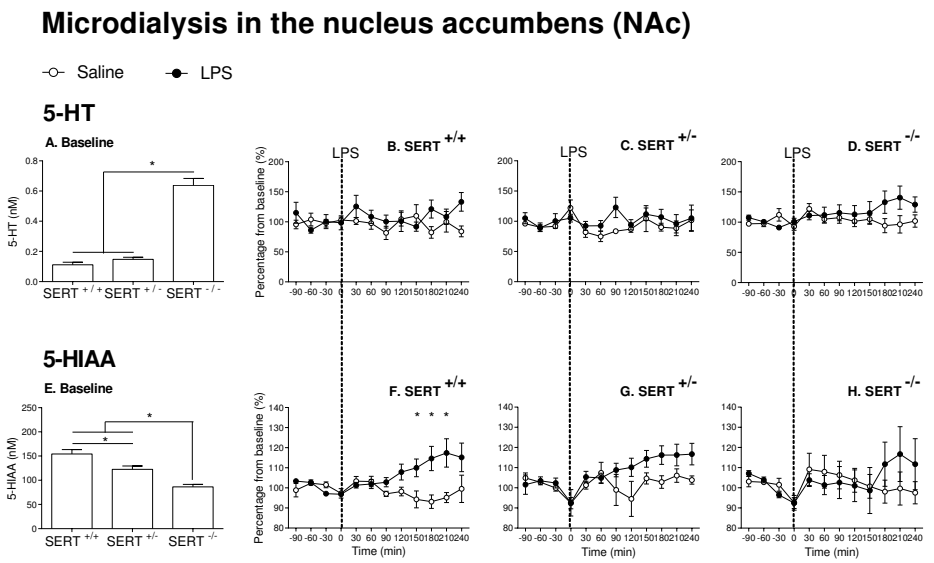

Figure 6.1: Extracellular 5-HT and 5-HIAA levels in the NAc of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats. Data was presented as mean ± standard error of the mean. Absolute mean baseline 5-HT and 5-HIAA levels are presented in the left column. Statistically significant differences between genotypes are indicated as * $p < 0.05$. For each genotype, the effect of a single LPS injection (i.p.) on extracellular accumbal 5-HT and 5-HIAA levels is presented as a percentage from baseline. Statistically significant differences between treatment groups are indicated as * $p < 0.05$.

The effect of peripheral LPS on 5-HT and 5-HIAA levels in the NAc and mPFC of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats

LPS did not affect 5-HT levels in the NAc and mPFC of $SERT^{+/+}$ (***Figure 6.1: B*** and ***Figure 6.2: B***, respectively) and $SERT^{+/-}$ rats (***Figure 6.1: C*** and ***Figure 6.2: C***, respectively). In $SERT^{-/-}$ rats, on the other hand, repeated measures ANOVA revealed a significant time x treatment interaction without an overall effect of treatment in both brain areas (NAc: time x treatment interaction: $F(3.1,27.6) = 4.0$, $p = 0.017$, $\varepsilon = 0.437$ (***Figure 6.1: D***) and mPFC: time x treatment interaction: $F(3.2,42.0) = 4.0$, $p = 0.012$, $\varepsilon = 0.416$ (***Figure 6.2: D***)). Post hoc analysis, however, did not reveal a significant difference at any time point.

Opposite effects were found for the serotonergic metabolite 5-HIAA. LPS increased extracellular 5-HIAA levels in the NAc and mPFC of $SERT^{+/+}$ rats (NAc: time x treatment interaction: $F(1.8,20.0) = 6.0$, $p = 0.011$, $\varepsilon = 0.260$ and treatment effect: not significant

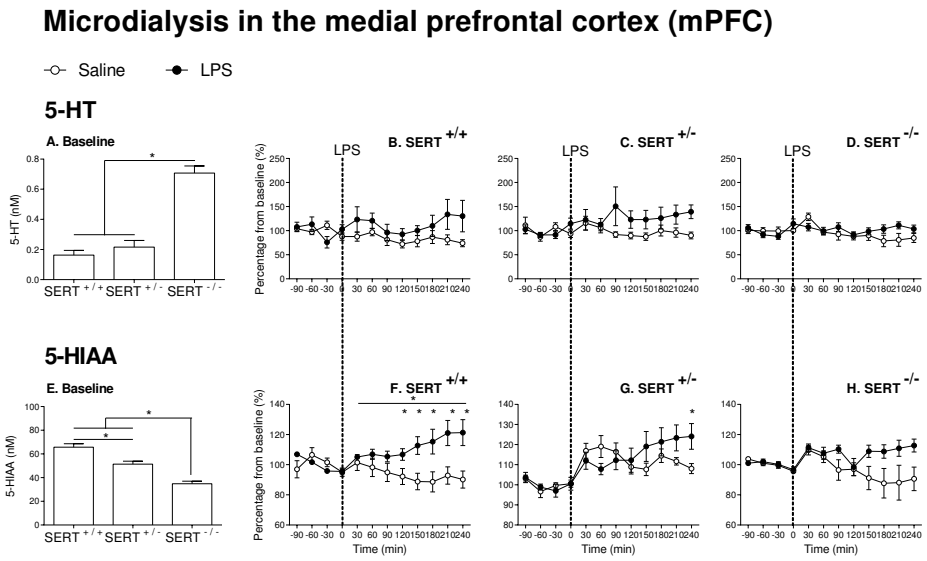

Figure 6.2: Extracellular 5-HT and 5-HIAA levels in the mPFC of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats. Data was presented as mean ± standard error of the mean. Absolute mean baseline 5-HT and 5-HIAA levels are presented in the left column. Statistically significant differences between genotypes are indicated as * $p < 0.05$. For each genotype, the effect of a single LPS injection (i.p.) on extracellular prefrontal 5-HT and 5-HIAA levels is presented as a percentage from baseline. Statistically significant differences between treatment groups are indicated as * $p < 0.05$.

(***Figure 6.1: F***); mPFC: time x treatment interaction: $F(2.0,23.6) = 6.3$, $p = 0.007$, $\varepsilon = 0.281$ and treatment effect: $F(1,12) = 6.2$, $p = 0.029$ (***Figure 6.2: F***)). Post hoc *t*-tests showed that accumbal 5-HIAA levels increased significantly at 150 min, 180 min and 210 min after exposure to LPS, whereas in the mPFC 5-HIAA levels were significantly increased at 120 min, 150 min, 180 min, 210 min and 240 min after exposure to LPS. Although $SERT^{+/-}$ rats showed no effect of LPS on 5-HIAA levels in the NAc (***Figure 6.1: G***), 5-HIAA levels in the mPFC increased significantly (***Figure 6.2: G***). There was a significant time x treatment interaction ($F(3.0,39.3) = 5.7$, $p = 0.002$, $\varepsilon = 0.432$) without an overall effect of treatment. Post hoc analysis revealed that 5-HIAA levels in the mPFC were significantly increased 240 min after exposure to LPS. $SERT^{-/-}$ rats did not show an LPS-induced 5-HIAA response. In the NAc (***Figure 6.1: H***) as well as in the mPFC (***Figure 6.2: H***) there was neither a time x treatment interaction nor an effect of treatment.

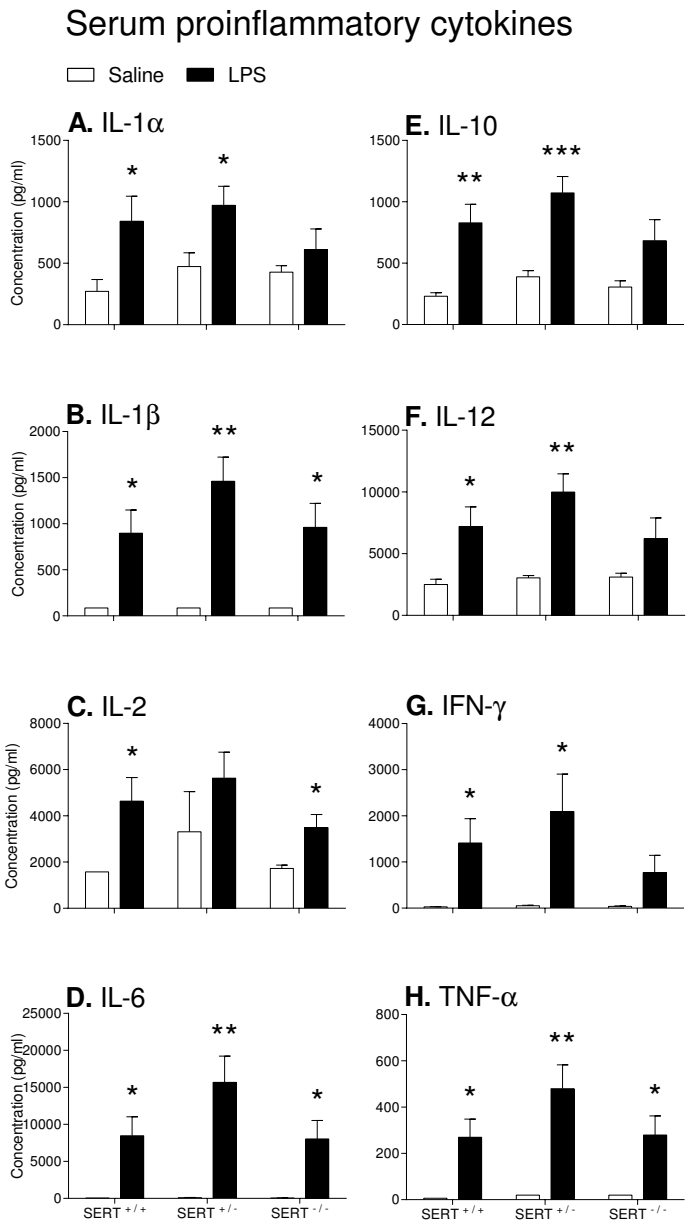

Figure 6.3: The effect of i.p. injected LPS on serum cytokine levels in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats (black bars) compared to saline injected $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats (white bars). Serum samples were collected 4 h after exposure to saline or LPS and in each sample IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-γ and TNF-α levels were measured with the Luminex. For each cytokine data was presented as mean ± standard error of the mean. For each genotype statistically significant differences between saline and LPS treated rats is indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Cytokine concentrations

The effect of peripheral LPS on serum cytokine levels of SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats

Except for IL-1α, IL-2, IL-10 and IL-12, serum cytokines were below detection limit in saline treated animals. However, after exposure to LPS, serum cytokine levels increased significantly. Although there was neither a significant genotype x treatment interaction nor a significant genotype effect, two-way ANOVAs revealed significant treatment effects for IL-1α ($F(1,41) = 12.3$, $p = 0.001$), IL-1β ($F(1,41) = 43.9$, $p < 0.001$), IL-2 ($F(1,41) = 7.6$, $p = 0.008$), IL-6 ($F(1,41) = 39.9$, $p < 0.001$), IL-10 ($F(1,41) = 32.5$, $p < 0.001$), IL-12 ($F(1,41) = 25.6$, $p < 0.001$), IFN-γ ($F(1,41) = 17.1$, $p < 0.001$) and TNF-α ($F(1,41) = 39.7$, $p < 0.001$) (***Figure 6.3***). Post hoc *t*-tests per genotype revealed significant increases for each cytokine in each genotype (at least $p < 0.05$), except for IL-1α, IL-10, IL-12 and IFN-γ in SERT$^{-/-}$ rats and IL-2 in SERT$^{+/-}$ rats.

GM-CSF and IL-4 serum levels were below detection limit in each genotype and treatment group (data not shown). Therefore these cytokines were excluded from data analysis.

The effect of peripheral LPS on forebrain cytokine levels of SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats

Two-way ANOVAs revealed significant treatment effects for forebrain IL-1α ($F(1,41) = 7.6$, $p = 0.009$) and IL-1β ($F(1,41) = 9.1$, $p = 0.004$) without significant genotype x treatment interactions and genotype effects. Post hoc *t*-tests per genotype, however, only revealed significant LPS-induced increases in forebrain IL-1α (saline: 90.7 pg/ml ± 2.7 and LPS: 103.4 pg/ml ± 3.4, $p = 0.011$) and IL-1β (saline: 150.4 pg/ml ± 4.4 and LPS: 175.0 pg/ml ± 10.6, $p = 0.035$) in SERT$^{+/-}$ rats. In comparison to the LPS-induced increases in serum cytokines, this increase in forebrain IL-1α and IL-1β was marginal. Furthermore, forebrain levels of GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α did not increase after exposure to LPS.

Discussion

The aim of the present study was to investigate the underlying mechanisms of our previous finding that SERT$^{-/-}$ rats are less susceptible to develop LPS-induced anhedonia than SERT$^{+/+}$ and SERT$^{+/-}$ rats, as measured by decreased sensitivity for brain stimulation

reward (van Heesch et al., 2013a). In the present study it is shown that SERT$^{-/-}$ rats do not differ in LPS-induced cytokine release as compared to SERT$^{+/+}$ and SERT$^{+/-}$ rats. The microdialysis study, on the other hand, shows that LPS-induced increases in accumbal and prefrontal 5-HIAA levels are only significantly reduced in SERT$^{-/-}$ rats.

LPS-induced increases in brain 5-HIAA levels do not appear in SERT$^{-/-}$ rats

Under basal conditions *total* 5-HT and 5-HIAA levels have been shown to be significantly lower in SERT$^{-/-}$ than in SERT$^{+/+}$ rats in several brain areas (Homberg et al., 2007). Interestingly, we and others have found that *extracellular* 5-HT levels are significantly increased in SERT$^{-/-}$ compared to SERT$^{+/+}$ and SERT$^{+/-}$ rats and mice (Homberg et al., 2007; Mathews et al., 2004; Olivier et al., 2008b), whereas extracellular levels of 5-HIAA were significantly decreased. Reductions in basal extracellular 5-HIAA levels have also been found in SERT$^{-/-}$ mice (Mathews et al., 2004). Due to the absence of SERT in SERT$^{-/-}$ rats, 5-HT cannot be transported back into the serotonergic neuron by SERT for storage into synaptic vesicles or breakdown by MAO into 5-HIAA. Possibly, that is why SERT$^{-/-}$ rats have less 5-HT and 5-HIAA in total and higher levels of 5-HT in the extracellular space. This shows that SERT plays a pivotal role in the regulation of serotonergic neurotransmission, but also in the formation of 5-HIAA.

Although SERT activity is completely absent in SERT$^{-/-}$ rats, formation of 5-HIAA is still present, possibly because alternative routes can lead to reuptake of 5-HT and corresponding 5-HIAA formation as well. Indeed, it has been demonstrated that the maximum rate (V_{max}) of 5-HT uptake in the hippocampus is reduced by 13.4% in SERT$^{+/-}$ rats and only by 72.2% in SERT$^{-/-}$ rats. Interestingly, inhibition of noradrenalin transporters (NET), but not dopamine transporters (DAT), attenuated the reuptake of the remaining 5-HT in the hippocampus of SERT$^{-/-}$ rats (Homberg et al., 2007). Furthermore, dopaminergic neurons in the VTA and substantia nigra have been shown to take up 5-HT by DAT in SERT$^{-/-}$ mice (Zhou et al., 2002). Although it has been demonstrated that NET and DAT concentrations were not different between genotypes throughout the brain (Homberg et al., 2007), this suggests that NET and DAT play an important role in the homeostasis of 5-HT in SERT$^{-/-}$ animals and that 5-HIAA levels in SERT$^{-/-}$ rats most probably originate from 5-HT reuptake by NET and DAT.

LPS led to a significant increase in extracellular 5-HIAA levels in the mPFC and NAc of SERT$^{+/+}$ rats. This has been described previously in rats and mice (Borowski et al., 1998; Prins et al., 2011; van Heesch et al., 2012). Furthermore, cytokines such as IL-1β and TNF-α, known to be released in response to LPS, have been shown to increase extracellular 5-HIAA levels in these brain areas as well (Merali et al., 1997; van Heesch et al., 2013b). SERT$^{-/-}$ rats, on the other hand, did not show an LPS-induced increase in 5-HIAA in both the mPFC and NAc. Since SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats have been shown to not

significantly differ in tryptophan levels, TRP/∑LNAA ratios, tryptophan hydroxylase activity and monoamine oxidase A activity (Homberg et al., 2007; Olivier et al., 2008a), we hypothesize that the inability of $SERT^{-/-}$ rats to significantly increase extracellular 5-HIAA levels in response to LPS is caused by the absence of SERT and therefore the inability to increase SERT activity and increase 5-HT metabolism in response to LPS. Indeed it has been shown that LPS and proinflammatory cytokines increase SERT activity (Mossner et al., 1998; Ramamoorthy et al., 1995; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010). It has been proposed that this process is IL-1 and p38 MAPK dependent (Zhu et al., 2004; Zhu et al., 2005; Zhu et al., 2010). It has been shown in mice, albeit not in rats, that the midline raphe nuclei express interleukin-1 receptors (IL-1R) (Cunningham et al., 1992) and that IL-1β- and LPS-induced increased SERT activity and LPS-induced immobility in the tail suspension test, a measure of behavioral despair, were abolished in IL-1R knockout mice (Zhu et al., 2010). Moreover, inhibition of the p38 MAPK signaling pathway with SB203580 blocked IL-1β-induced stimulation of SERT (Zhu et al., 2006). This suggests that the IL-1 signaling pathway is pivotally involved in the development of LPS-induced anhedonia via a p38 MAPK-dependent pathway (Baganz and Blakely, 2013). Further indications for an important role for increased SERT activity in LPS-induced anhedonia arises from clinical research showing that LPS-induced depressive symptoms could be reduced by 5-day pretreatment with the SSRI citalopram (Hannestad et al., 2011b). Besides, there are indications that prophylactic administration of SSRIs can be successfully used in a subgroup of patients who are at risk of developing major depression during IFN-α treatment (Galvao-de Almeida et al., 2010).

LPS-induced cytokine release in serum and brain is not affected in $SERT^{-/-}$ rats

As expected, serum cytokines increased significantly after exposure to LPS. In contrast, forebrain cytokines were not affected by LPS, or in case of IL-1α and IL-1β, only increased marginally. Previously with immunohistochemistry, however, dramatic local increases in IL-1β in circumventricular organs (CVOs) have been found after peripheral exposure to LPS (Konsman et al., 1999). Furthermore, it has been shown that IL-1β is involved in LPS-induced behavioral changes, such as reductions in social interaction and increases in behavioral despair (Konsman et al., 2008; Zhu et al., 2010). In the present experiment, the forebrain was taken to measure cytokine concentrations, thus the effect might have been diluted if IL-1β was mainly present in the CVOs. Therefore, we think that these small increases in forebrain IL-1α and IL-1β still could be biologically relevant.

Although there were significant effects of treatment, no effects of genotype were found. This demonstrates that the used dose of LPS effectively activated the peripheral immune system in each genotype and that $SERT^{-/-}$ rats do not differ from $SERT^{+/+}$ and $SERT^{+/-}$ rats in their immune response. This is in accordance with the observation that chronic

pretreatment of rats with fluoxetine or imipramine (SSRIs) did not affect LPS-induced increases in spleen IL-1β mRNA and spleen TNF-α mRNA (Yirmiya et al., 2001) and that 5-day treatment with citalopram (SSRI) reduced LPS-induced depressive symptoms in humans without affecting the TNF-α and IL-6 response (Hannestad et al., 2011b). Furthermore, it has been demonstrated *ex vivo* that chronic treatment with fluoxetine (SSRI) significantly reduced the increased SERT and IFN-γ mRNA expression levels in peripheral blood mononuclear cells of depressed patients but not IL-1β, TNF-α and IL-6 mRNA expression levels compared to healthy controls. Interestingly, this was associated with clinical improvement (Tsao et al., 2006). Additionally, the latter study revealed a positive correlation between SERT and cytokine mRNA expression, further suggesting that high levels of proinflammatory cytokines and increased SERT activity are involved in the pathophysiology of anhedonia and depression (Tsao et al., 2006). However, as mentioned previously in the introduction, SSRIs have also been shown to suppress cytokine production by T_{H1} cells in whole blood *ex vivo* (Diamond et al., 2006; Taler et al., 2007) and decrease serum proinflammatory cytokine levels in depressed patients (Hannestad et al., 2011a), suggesting that inhibition of SERT leads to a reduced immune response. However, the present serum and forebrain cytokine profiles show that the LPS-induced release of cytokines was not affected by SERT and therefore reduced cytokine release in response to LPS could not explain the reduced susceptibility of $SERT^{-/-}$ rats to LPS-induced anhedonia.

Conclusions

In conclusion, the present study supports the hypothesis that increased SERT function is important in LPS-induced anhedonia. We showed that LPS-stimulated cytokine release in serum and brain is independent of SERT, whereas LPS-induced 5-HIAA formation is dependent of SERT. It is hypothesized that LPS-induced cytokines increase SERT activity as demonstrated by increased 5-HIAA formation. Since SERT is absent in $SERT^{-/-}$ rats, LPS-induced proinflammatory cytokines cannot increase SERT activity and therefore LPS-induced anhedonia, as previously shown by us (van Heesch et al., 2013a), and subsequent LPS-induced increases of 5-HIAA are significantly reduced in these animals.

Acknowledgements

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7

General discussion

Studying the role of monoamine transporters in inflammation-induced anhedonia

The notion that peripheral inflammations affect our mood is gaining in credence. It has been hypothesized that immune activation, via the release of peripheral and brain cytokines, may be involved in the etiology and symptomatology of "depression due to a general medical condition" (Yirmiya et al., 1999; Yirmiya et al., 2000). Understanding the mechanisms leading to inflammation-induced depression may lead to new therapeutic targets for treatment of this subgroup of depressed patients. The focus of this thesis is the investigation of the role of monoamine transporters in inflammation-induced anhedonia. In order to do this, intracranial self-stimulation (ICSS) studies (**Chapter 2, 3 and 5**) and *in vivo* microdialysis studies (**Chapter 2**, **3**, **4 and 6**) were performed to measure inflammation-induced anhedonia and inflammation-induced alterations in monoamine and monoamine metabolite levels in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), respectively. In this final chapter, the results of the experiments that are presented in this thesis are summarized and discussed. Furthermore, suggestions are given for future research in the field of inflammation-induced depression and the significance of the data presented in this thesis for patients diagnosed with "depression due to a general medical condition" is evaluated.

LPS and ICSS as a model for inflammation-induced anhedonia

Lipopolysaccharide (LPS) is widely used in biomedical research to activate the innate immune system of laboratory animals. In **Chapter 2** and **Chapter 4** it was investigated whether the doses used of LPS in our experiments lead to anhedonia, i.e. the inability to experience pleasure. The ICSS procedure was used to measure brain reward function in rats and mice during a peripheral LPS challenge and it was demonstrated that LPS decreased brain stimulation reward significantly in both rats (250 µg/kg) (**Chapter 2**) and mice (133 µg/kg) (**Chapter 4**). Interestingly, in both species, most significant decreases in responding for rewarding brain stimulation were found 4 h after exposure to LPS. At this time point serum cytokine levels are strongly increased and at their peak (Biesmans et al., 2013; Dantzer et al., 2008). This suggests that LPS-induced cytokines are responsible for decreased brain stimulation reward as measured in the ICSS procedure. Indeed, in **Chapter 6**, a significant increase in serum and brain cytokines was observed in serotonin transporter wild type rats ($SERT^{+/+}$) 4 h after LPS. In concurrence, severity of LPS-induced depression is highly correlated with blood levels of cytokines in humans exposed to LPS (Yirmiya et al., 2000). One of the cytokines that is highly increased by LPS is tumor necrosis factor α (TNF-α). In **Chapter 3** it is shown that TNF-α leads to, although less significant than LPS, a significant decrease in responding for rewarding brain stimulation as well. In addition to TNF-α, other investigators have shown that IL-2 also reduces brain stimulation reward

(Anisman et al., 1996; Anisman et al., 1998). IL-1β and IL-6, on the other hand did not affect brain stimulation reward (Anisman et al., 1998), suggesting that not every single cytokine is able to affect the reward system. However, we cannot exclude that IL-1β and IL-6 are able to affect brain stimulation reward in concert with other proinflammatory cytokines, such as TNF-α. Nevertheless, the data suggest that there is an important role for proinflammatory cytokines in mediating the response that is responsible for LPS-induced decreases in brain stimulation reward.

Before we can conclude that LPS and TNF-α induced decreases in brain stimulation reward reflect of state of anhedonia, one should exclude that deficits in motor functions could have negatively affected the capability to perform the ICSS task. This is of special importance in case of LPS and TNF-α, since it is well-known that activation of the immune system by LPS reduces distance moved in the open field (**Chapter 2** and O'Connor et al., 2009), suggesting that motor activity is impaired by LPS and possibly also by TNF-α. In **Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5**, however, it is shown that response latencies, i.e. the time that elapses between the delivery of the electrical stimulus and the turning of the wheel manipulandum for all of the trials that led to a positive response, did not increase after exposure to LPS or TNF-α. In fact, LPS even showed to decrease response latencies in both rats (**Chapter 2** (trend) and **Chapter 5**) and mice (**Chapter 4**), indicating that LPS-treated animals responded even faster to the non-contingent stimulus compared to saline treated animals. It is known that saline-treated animals spend the intertrial time in the ICSS procedure moving around (Markou and Koob, 1992). In contrast, LPS-treated rats and mice become immobile and position themselves next to the wheel (personal observation). Therefore, animals exposed to LPS can respond faster to the non-contingent stimulus, because these animals do not have to orient themselves towards the wheel after delivery of the non-contingent stimulus. Nevertheless, most importantly, the response latency data show that reduced motor performance is not responsible for the observed increases in ICSS thresholds after exposure to LPS or TNF-α. Indeed, it has previously been demonstrated that performance effects are primarily reflected by increased response latencies, whereas anhedonia is primarily reflected by increased ICSS thresholds, i.e. decreased brain stimulation reward (Markou and Koob, 1992).

Besides impairing motor activity, activation of the immune system is known to reduce appetite (Langhans and Hrupka, 1999; Langhans, 2007) leading to reductions in body weight. Indeed, in **Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5** it is shown that both LPS and TNF-α significantly reduce body weight of rats and mice. Again this shows that LPS-induced cytokines, such as TNF-α, are probably responsible for behavioral changes observed in sick animals. In contrast to the sucrose preference test, which is another commonly used rodent model to evaluate anhedonia, ICSS has the strong advantage that it is independent of appetite and food intake (Carlezon and Chartoff, 2007; Spies, 1965).

Therefore, we think that, in the field of inflammation-induced depression research, ICSS is the most accurate method to assess anhedonia.

According to the data presented in this thesis, highest levels of anhedonia were found 4 h after exposure to LPS and TNF-α in both rats and mice. At this time point, levels of proinflammatory cytokines and symptoms of sickness behavior are known to be at their highest levels as well (Biesmans et al., 2013; Dantzer et al., 2008). In contradiction to our findings, however, it has previously been hypothesized that LPS-induced depression-like behavior in rodents gradually develops during sickness behavior, reaching its peak 24 h after exposure to LPS, whereas symptoms of sickness behavior are already returning to baseline levels at that time point (Dantzer et al., 2008). According to **Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5**, it can be hypothesized that cytokine levels, anhedonia and other symptoms of sickness behavior increase simultaneously, reaching their peak 2 to 6 h after LPS. However, in rats (**Chapter 2** and **Chapter 5**), but not in mice, it is shown that anhedonia is still present 24 h after exposure to LPS, suggesting that symptoms of anhedonia persist longer compared to other symptoms of sickness behavior (***Figure 7.1***).

Since reward and pleasure are highly interconnected and response latencies were not increased by LPS and TNF-α, this strongly suggests that LPS- and TNF-α-induced reductions in brain stimulation reward reflect a state of anhedonia. The same doses of LPS and TNF-α that were used in the ICSS studies were used in the microdialysis studies as well. This was done in order to investigate how LPS and TNF-α affect monoamine signaling in the brain and how monoamine transporters are involved in these inflammation-induced changes in monoamine signaling (**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 6**).

Inflammation-induced anhedonia is not associated with monoamine depletions in the synaptic cleft

Antidepressants currently available predominantly improve depression by interfering with monoamine signaling in the brain leading to increased extracellular levels of monoamines. This indicates that central monoamines are important regulators of emotional behavior and therefore it is interesting to know how LPS and TNF-α, both known to induce anhedonia (**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5**), affect monoamine signaling in the brain. Therefore, in **Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 6** microdialysis studies were performed to investigate how a peripheral injection with LPS or TNF-α affects extracellular monoamine (serotonin (5-HT) and dopamine (DA)) and monoamine metabolite levels (5-HIAA, DOPAC and HVA) in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC).

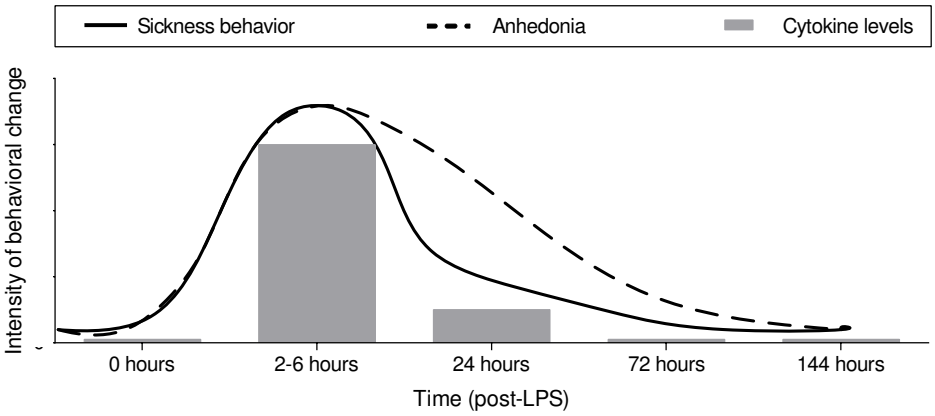

Figure 7.1: Hypothetical picture of the development of LPS-induced anhedonia and sickness behavior in rats (adjusted picture after Dantzer et al., 2008). Intraperitoneally injected LPS initiates a cytokine response that peaks 2 to 6 h later and gradually diminishes. During this cytokine peak, sickness behavior develops and diminishes once cytokine levels decrease. Anhedonia, as measured by the intracranial self-stimulation procedure, originates and peaks at the same time as the other symptoms of sickness behavior, but persists longer.

The NAc is intensely innervated by dopaminergic neurons arising from the VTA. Since drugs of abuse, e.g. nicotine, cocaine and amphetamine, are associated with pleasure (reward) and high levels of DA in the NAc (Di Chiara and Imperato, 1988), special attention was given to DA levels in the NAc in response to LPS and TNF-α. It was hypothesized that LPS-induced anhedonia would be associated with decreased DA levels in the NAc. Surprisingly, however, a trend towards a significant treatment effect for both LPS and TNF-α was observed. This suggests that activation of the immune system leads to increased DA levels instead of decreased DA levels in the NAc (**Chapter 2**, **Chapter 3** and **Chapter 4**). This is in concurrence with LPS-induced increases (Borowski et al., 1998), but in contradiction with IL-2-induced decreases of DA in the NAc of rats (Anisman et al., 1996). Remarkably, the same doses of LPS and IL-2 have been demonstrated to reduce responding for rewarding brain stimulation in the ICSS procedure (Anisman et al., 1996; Borowski et al., 1998). This shows that increased DA levels in the NAc are not necessarily associated with pleasure/hedonia and that anhedonia is not necessarily associated with decreased DA levels in the NAc. Since stress leads to increased accumbal DA levels in the extracellular space as well and LPS and TNF-α, but not IL-2, are able to increase HPA-axis activity (Dunn, 2000), it is hypothesized that increased HPA-axis activity is responsible for

LPS- and TNF-α-induced trends towards increased levels of DA in the NAc. Besides increasing DA signaling in the NAc, physical and physiological stressors also increase efflux of prefrontal DA. Although it has to be mentioned that 'immune stressors', such as LPS, lead to smaller increases in extracellular prefrontal DA levels compared to physical and physiological stressors (Dunn, 2006), it is not surprising that peripheral LPS significantly increased prefrontal DA levels in both mice and rats (**Chapter 2** and **Chapter 4**). The latter is in correspondence with another LPS microdialysis study performed in rats (Dunn, 2006).

Serotonin reuptake inhibitors (SSRIs) are the most prescribed antidepressants (Stafford et al., 2001) known to elevate mood and to interfere with 5-HT signaling in the brain. Nevertheless, LPS and TNF-α did not affect extracellular levels of this monoamine in the NAc and mPFC (**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 6**). Therefore, LPS- and TNF-α-induced anhedonia is not necessarily associated with monoamine depletion in the synaptic cleft as measured by microdialysis. In concurrence, a meta-analysis study revealed that depletions of 5-HT and DA/NE do not lead to depression in healthy subjects (Ruhe et al., 2007). Another explanation can be that short-term decreases (or increases) in monoamine release cannot be detected by the present microdialysis procedure (Stenfors and Ross, 2004).

Inflammation-induced anhedonia is associated with increased formation of monoamine metabolites

Extracellular levels of 5-HIAA (metabolite of 5-HT), DOPAC and HVA (metabolites of DA), were measured in the NAc and mPFC. Interestingly, in both mice and rats, these metabolites increased significantly after peripheral exposure to LPS and TNF-α (**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 6**). Since TNF-α has similar effects as LPS, although less powerful, it is suggested that LPS-induced cytokines, such as TNF-α, are responsible for these LPS-induced increases in monoamine metabolite levels.

Monoamine oxidase (MAO) is responsible for the deamination of 5-HT and DA into 5-HIAA and DOPAC, respectively (Deutch and Roth, 2008; Eisenhofer et al., 2004). This enzyme is located in monoaminergic neurons on the outer membrane of mitochondria. HVA, on the other hand, is formed by the enzyme COMT. Therefore, increased MAO activity and COMT activity are responsible for increased formation of 5-HT/DOPAC and HVA, respectively. However, before MAO has access to 5-HT and DA, 5-HT and DA should be present in the neuroplasm. Therefore, the different sources of increased 5-HIAA and DOPAC levels are: 1) increased formation of 5-HT and DA leading to increased 5-HT and DA that can be metabolized before it is taken up by storage vesicles; 2) decreased vesicular monoamine transporter (VMAT) activity leading to decreased storage of 5-HT and DA into storage vesicles; 3) increased leakage of 5-HT and DA from storage vesicles;

and 4) increased monoamine transporter activity leading to increased 5-HT and DA that can be metabolized before it is taken up by storage vesicles (Stenfors and Ross, 2004). The focus of this thesis was on monoamine transporters in inflammation-induced anhedonia.

Inflammation-induced anhedonia is associated with increased monoamine transporter activity

The observation that peripheral LPS (**Chapter 2**) and TNF-α (**Chapter 3**) resulted in increased metabolite formation in the NAc and mPFC, changed the focus towards the role of monoamine transporters in LPS-induced anhedonia. Since 5-HIAA and DOPAC are predominantly formed after reuptake by monoamine transporters (Stenfors and Ross, 2004), it is hypothesized that increased monoamine transporter activity is responsible for this significant increase in monoamine metabolite levels (see **Chapter 1** section: "Monoamine transporters"). In **Chapter 4** it was investigated whether DOV 216,303, a triple reuptake inhibitor (TRI) (Skolnick et al., 2006), was able to inhibit LPS-induced monoamine metabolite formation in the NAc and mPFC of mice. Previously, 5 mg/kg peripherally injected DOV 216,303 has been shown to effectively, but not completely, inhibit SERT, DAT and NET activity in the mPFC of mice (Caldarone et al., 2010). Interestingly, here it was shown that partial inhibition of SERT, DAT and NET by 5 mg/kg DOV 216,303, led to complete abolishment of LPS-induced increases in DOPAC and HVA in the NAc and mPFC. This suggests that LPS-induced DAT and NET activity are responsible for LPS-induced increases in dopaminergic metabolites in the NAc and mPFC, respectively. Since DAT density is very low in the mPFC (Sesack et al., 1998), DA is taken up by the available NET (Gresch et al., 1995; Gu et al., 1994) and therefore increased DOPAC formation is predominantly the result of increased NET activity (see **Chapter 1**, ***Figure 1.1***). Furthermore, it was shown that LPS-induced 5-HIAA formation was abolished by DOV 216,303 in the mPFC, suggesting that LPS increases SERT activity in this brain structure as well. In the NAc, on the other hand, DOV 216,303 delayed the LPS-induced formation of 5-HIAA, but there was still a significant increase of 5-HIAA. It is worth noting that in contrast to the mPFC, the NAc is just poorly innervated by serotonergic neurons arising from the raphe nucleus (Molliver, 1987). This might explain why DOV 216,303 was less successful in inhibiting LPS-induced 5-HIAA formation in the NAc compared to the mPFC.

In literature, most data point to an important role for increased SERT activity in LPS- and proinflammatory cytokine-induced depression-like behavior. It has already been shown that proinflammatory cytokines increase SERT activity, probably via activation of the p38 MAPK signaling pathway (Mossner et al., 1998; Ramamoorthy et al., 1995; Tsao et al., 2008; Zhu et al., 2006; Zhu et al., 2010). Indeed, increased SERT activity could explain why we found increased levels of 5-HIAA after exposure to LPS that is blocked by

pretreatment with DOV 216,303 (**Chapter 4**). Less is known, however, about the effect of proinflammatory cytokines on DAT and NET activity. For instance, it has been shown *ex vivo* that peripheral LPS increases NET activity but not DAT activity in midbrain synaptosomes (Zhu et al., 2010) and that 7-day pretreatment with bupropion (a NET and DAT inhibitor), did not inhibit LPS-induced anhedonia in healthy subjects (DellaGioia et al., 2013). In other studies, however, it has been demonstrated that LPS and proinflammatory cytokines stimulate DAT activity in the brain (Moron et al., 2003; Nakajima et al., 2004). Since it is also shown in **Chapter 4** that DOV 216,303 pretreatment significantly reduced formation of LPS-induced DOPAC and HVA formation in the NAc and mPFC, suggesting that activation of the immune system by peripheral LPS increases both DAT and NET activity, it is argued that more research into the possible role of DAT and NET in inflammation-induced anhedonia is necessary.

SERT activity is necessary to provoke LPS-induced anhedonia

The next question that arose was: 'Is LPS-induced increased monoamine transporter activity necessary for the development of anhedonia?' Therefore, in **Chapter 5** an ICSS study was performed in serotonin transporter knockout ($SERT^{-/-}$) rats to investigate how these animals respond to an LPS challenge. $SERT^{-/-}$ rats are known to be more sensitive to stress and to display more anxious and depression-like behavior in several behavioral tests (Olivier et al., 2008b). Knowing that LPS-induced cytokines are able to activate the HPA-axis (Dunn, 2000) it could be expected that $SERT^{-/-}$ rats are more sensitive to LPS-induced anhedonia as well. However, the opposite result was found: in contrast to SERT wild type ($SERT^{+/+}$) rats, LPS did not significantly decrease brain stimulation reward in $SERT^{-/-}$ rats. Animals heterozygous for the SERT ($SERT^{+/-}$rats) showed results that were in between the data of $SERT^{+/+}$ and $SERT^{-/-}$ rats. This suggests that a dose-response mechanism is involved with higher increases in SERT activity resulting in increased sensitivity for LPS-induced anhedonia. In agreement with this ICSS study it has previously been demonstrated that $SERT^{-/-}$ mice show reduced LPS-induced depression-like behavior in the tail suspension test (Zhu et al., 2010). Altogether, this suggests that there is an important role for increased monoamine transporter activity in inflammation-induced anhedonia.

Besides the abolishment of LPS-induced anhedonia, body weight of $SERT^{-/-}$ rats decreased less dramatically compared to $SERT^{+/+}$ rats upon the LPS-challenge. Furthermore, before exposure to LPS, body weight of $SERT^{-/-}$ rats was significantly lower compared to $SERT^{+/+}$ rats. This suggests that body weight is under influence of SERT as well. Indeed, since 5-HT is well-known to influence appetite negatively (Hutson et al., 1988; Schwartz et al., 1989) and extracellular levels of 5-HT are significantly higher in $SERT^{-/-}$ rats (**Chapter 6** and Homberg et al., 2007; Olivier et al., 2008b), the lower basal body weight in $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats was not surprising.

Increased SERT-activity is involved in 5-HIAA formation but not in cytokine release

The question that arose based on the results of the ICSS study in SERT$^{-/-}$ rats was 'Why are SERT$^{-/-}$ rats less sensitive for LPS-induced anhedonia?' Two factors might have contributed to this protection. First of all, SSRIs have been shown to decrease levels of proinflammatory cytokines in depressed patients (Hannestad et al., 2011a; Sutcigil et al., 2007). This suggests that inhibition of SERT is involved in dampening of the cytokine response and therefore release of proinflammatory cytokines in response to LPS could be lower in SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats. Secondly, increased SERT activity itself, as demonstrated by increased 5-HIAA formation, could be the cause of LPS-induced anhedonia. To this end cytokines were measured in serum and brain 4 h after exposure to peripheral LPS. The results of this study, presented in **Chapter 6**, showed that SERT$^{-/-}$ rats did not differ in their immune response compared to SERT$^{+/+}$ and SERT$^{+/-}$ rats. This is in agreement with the observation that 5-day pretreatment with citalopram (SSRI) reduced LPS-induced depressive symptoms in human without affecting the TNF-α and IL-6 response (Hannestad et al., 2011b). The microdialysis data, on the other hand, showed that LPS-induced increases in 5-HIAA were abolished in SERT$^{-/-}$ rats, whereas they did appear in SERT$^{+/+}$ and to a lesser extent in SERT$^{+/-}$ rats. This shows that SERT activity plays a pivotal role in the formation of 5-HIAA and furthermore it suggests that increased SERT activity is necessary to provoke LPS-induced anhedonia.

Thus, based on the data presented in this thesis, it is concluded that increased monoamine transporter activity and predominantly increased SERT activity, is involved in the onset of inflammation-induced anhedonia.

Future perspectives and limitations

To get a better insight in the mechanisms behind inflammation-induced depression, a single intraperitoneal LPS or TNF-α injection was used to activate the immune system for a short period (**Chapter 2 till Chapter 6**). Subsequently, this led to a temporary decrease in responding to the rewarding effects of selfstimulation in the ICSS procedure (**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5**). Since patients are diagnosed with depression only when symptoms of depression are present for at least 2 weeks (APA, 1994), it would be more interesting to measure anhedonia in an animal model with long-term high serum proinflammatory cytokine levels. It has already been shown that repeated exposure to LPS leads to tolerance to the anhedonic effects of LPS (Barr et al., 2003). Therefore, we propose that long-term activation of the immune system should be studied in an animal model for a chronic disease that is known to activate the immune system for a longer period, for instance in a tumor model or inflammatory bowel disease model. It is hypothesized that chronic activation of the immune system will reduce brain stimulation reward for a long

period. Once such a model is validated, antidepressants and other treatment forms (see section: "Clinical significance and recommendations") could be tested to investigate which pharmacological treatments are potentially useful to diminish proinflammatory cytokine-induced anhedonia in patients diagnosed with "depression due to a general medical condition". Based on the results in this thesis, it would be interesting to start with an SSRI, for instance escitalopram, with or without bupropion (a selective NET and DAT inhibitor) or a TRI, for instance DOV 216,303 (not available for clinical use). Although here only short-term effects of inflammation-induced anhedonia were studied, making acute administration of LPS and TNF-α a less attractive translational model for inflammation-induced depression in humans, the present experiments contributed to a better insight in the onset of inflammation-induced anhedonia. As such, in the present thesis it is shown that anhedonia is rapidly induced by cytokines that originated from the periphery (within 1 h) and that this is associated with increased levels of monoamine metabolites in the NAc and mPFC, suggesting that proinflammatory cytokines increase monoamine transporter activity.

SSRIs are known to elevate mood in depressed patients. Nonetheless, their effectiveness in relieving symptoms of anhedonia has been questioned (McCabe et al., 2010). In our studies, however, we showed that inhibition of SERT by pretreatment with DOV 216,303 (**Chapter 4**) and lifelong absence of SERT (**Chapter 6**) prevented LPS-induced increases in accumbal and prefrontal 5-HIAA, suggesting that SERT activity is increased after activation of innate immunity. Furthermore, it is shown that lifelong absence of SERT abolished LPS-induced anhedonia (**Chapter 5**), demonstrating an important role for increased SERT activity in LPS-induced anhedonia. In this thesis a 'prophylactic' treatment was investigated, whereas for patients with "depression due to a general medical condition" it would be more interesting to find a successful symptomatic treatment. Therefore it would be important to investigate in animal models, as described above in this section, whether SSRIs are able to reduce anhedonia after chronic activation of the immune system. For humans, it has already been demonstrated in meta-analysis studies that prophylactic as well as symptomatic treatment of IFN-α-induced depression with SSRIs for the treatment of chronic hepatitis C virus infection successfully decreased the incidence of IFN-α-induced depression (Jiang et al., 2013; Sockalingam and Abbey, 2009), suggesting that SSRIs can be successfully used in inflammation-induced depression.

In order to get a better insight in the role of SERT in inflammation-induced depression, LPS-induced anhedonia and alterations in monoamine signaling were studied in $SERT^{-/-}$ rats (**Chapter 5** and **Chapter 6**). Since 5-HT acts as a neurotrophic factor during neuronal development (Gaspar et al., 2003), the absence of SERT in $SERT^{-/-}$ rats might have interfered with neuronal development, which consequently might have influenced the behavioral response to LPS in these animals. In a pilot study, however, it was shown that LPS-induced anhedonia is partially inhibited in rats that were pretreated with escitalopram (SSRI) for 2 weeks (unpublished data), suggesting that inactivity of SERT during the LPS

challenge, and not differences in neurodevelopment, is responsible for the lack of LPS-induced anhedonia in SERT$^{-/-}$ rats. Nevertheless, it is recommended to repeat this SSRI experiment in a larger cohort.

In this thesis, the effect of peripheral immune activation on anhedonia and monoaminergic signaling was investigated in rats as well as in mice. We demonstrated that mice recover more rapidly from LPS-induced anhedonia than rats (**Chapter 2**, **Chapter 4** and **Chapter 5**). This difference in sensitivity to LPS between rats and mice is not surprising, since there are already marked differences in innate immune responses and sensitivity for LPS-induced depression-like behavior between mouse strains (Browne et al., 2012; Watanabe et al., 2004). Furthermore, we used a lower dose of LPS in mouse studies (133 µg/kg) compared to rat studies (250 µg/kg). Since LPS decreased responding for brain stimulation reward in both rats and mice, and both species showed the same LPS-induced alterations in monoamine signaling: i.e. increased formation of monoamine metabolites in the NAc and mPFC (**Chapter 2**, **Chapter 4** and **Chapter 6**), the underlying mechanisms seem to be similar in rats and mice and extrapolation of these mechanism to humans are therefore more reliable. Therefore, using both rats and mice is an added value instead of a limitation.

Clinical significance and recommendations

The results in the present thesis confirm that there is an important role for proinflammatory cytokines in anhedonia, i.e. a core symptom of major depression. Many infectious (common cold, influenza, herpes virus, cytomegalovirus, Epstein-Barr virus, gastroenteritis, Borna disease virus, HIV) and non-infectious diseases (autoimmune diseases, stroke, trauma, Alzheimer's disease and other neurodegenerative disorders) are associated with activation of the immune system, i.e. secretion of proinflammatory cytokines, and high prevalence's of depressive symptoms (Yirmiya et al., 2000). Therefore, inflammation-induced depression may be of particular importance in patients diagnosed with medical illnesses. Thus, medical practitioners should also be concerned about the mental health of patients that suffer from disorders that are associated with increased levels of proinflammatory cytokines. Since depression increases the level of disability above that of the level of disability experienced by the medical illness alone, it is reasonable to routinely screen for depression in patients that are diagnosed with a medical illness and start antidepressant treatment when necessary. Therefore, it is important to find an effective treatment for patients diagnosed with "depression due to a general medical condition". This thesis focused on the role of monoamine transporters in LPS-induced anhedonia. Principally the results obtained in **Chapter 5** and **Chapter 6** indicate that inhibition of SERT could protect patients from inflammation-induced anhedonia and possibly also inflammation-induced sickness behavior (reductions in body weight). Indeed, in rheumatoid arthritis animal models SSRIs significantly reduced arthritis compared to controls (Baharav

et al., 2012; Sacre et al., 2010), showing that SSRIs possess anti-inflammatory properties. Furthermore, it has been demonstrated that SSRIs can be effectively used to treat depression in patients with rheumatoid arthritis (Slaughter et al., 2002). However, in psoriatic patients, the SSRI fluoxetine, seem to induce psoriasis (Tamer et al., 2009; Tan Pei Lin and Kwek, 2010). Therefore, it is important to elucidate the effect of SSRIs in each specific general medical condition. We point out that SSRIs should not be used once it has been shown that the symptoms of the medical illness become even worse due to the antidepressant treatment.

As mentioned previously in the "General introduction", proinflammatory cytokines can induce changes in the brain via other pathways as well. Concerning this, there are several other options to treat "depression due to a general medical condition":

- According to literature and results presented in this thesis, cytokines seem to be responsible for LPS-induced sickness behavior, LPS-induced anhedonia and LPS-induced increases in monoamine transporter activity. This is evidently underlined by the observation that TNF-α antibodies effectively relieve symptoms of inflammatory diseases (Aaltonen et al., 2012; Menter et al., 2010; van Dullemen et al., 1995) and relieve accompanying symptoms of depression (Tyring et al., 2006; Uguz et al., 2009). Therefore, treatment for "depression due to a general medical condition" could also be directly directed towards inhibition of production/release of proinflammatory cytokines;
- Furthermore, treatments could target inflammatory signaling pathways. For instance combination therapy of cyclo-oxygenase (COX)1, COX2 inhibitors and antidepressants (NRI and SSRI) improved the efficacy of the antidepressants (Mendlewicz et al., 2006; Muller et al., 2006). Furthermore, omega-3 fatty acids have anti-inflammatory effects and have been shown to increase efficacy of SSRIs (Gertsik et al., 2012; Lin and Su, 2007). This suggests that inflammation-induced depression could be targeted via diet as well. Since inhibition of p38 MAPK has been shown to prevent the development of LPS-induced depression-like behavior in the tail suspension test, p38 MAPK inhibitors are another possibility to treat inflammation-induced depression (Zhu et al., 2010). Moreover, during inflammation NFkB is increased and NFkB inhibitors have been shown to have anti-inflammatory and antidepressant-like effects in animal models as well (Godbout et al., 2005; Lopresti et al., 2012);
- Concerning cytokine-induced degradation of BH4, monoamine synthesis could be improved by increasing BH4 activity. For instance by using BH4 supplements;
- Currently, IDO inhibitors are tested for efficacy in cancer and other diseases (Di Pucchio et al., 2010) and may be useful for treating inflammation-induced depression as well. Furthermore, pretreatment with ketamine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, effectively blocked LPS-induced

depressive-like behavior in mice (decreased sucrose preference and increased immobility in the forced swim test) (Walker et al., 2013).

All in all, there are many potential strategies for the treatment of inflammation-induced depression. Most of these treatments are still investigated in a pre-clinical stage. Therefore, it is recommended that large-scale, longitudinal, placebo-controlled, clinical trials are mandatory in order to identify the most relevant treatment strategies for patients diagnosed with "depression due to a general medical condition".

Although it seems reasonable that depressed patients diagnosed with a general medical condition that is associated with high levels of proinflammatory cytokines are the principle group of patients that can benefit from these new antidepressant therapies, we cannot exclude that these new treatment strategies could be beneficial for depressed patients without a chronic inflammatory condition as well. It is good to realize that under healthy conditions immune homeostasis is critically regulated. Hence, it is possible, that only a slight, but continuous disbalance can already increase the risk for developing major depression. Therefore, it is recommended to investigate effectiveness of these new treatment strategies in major depressed patients that do not respond to the regular treatment, without direct evidence for an underlying general medical condition as well.

Conclusions

In this thesis it is confirmed that activation of the immune system by LPS induces anhedonia in rats and mice. Furthermore, we showed that TNF-α, the most principle cytokine that mediates acute and innate inflammation and is highly upregulated after exposure to LPS, induced anhedonia in mice as well. Therefore, we underline the importance of screening for major depression in chronic inflammatory conditions. Furthermore, it is demonstrated that in particular increased SERT activity is important in inflammation-induced anhedonia. This offers opportunities for the treatment of depression that is associated with high levels of proinflammatory cytokines. Monoamine transporter activity is not the only route by which proinflammatory cytokines could induce a depression. Therefore, further research is needed to establish all the mechanisms involved in inflammation-induced depression in order to find effective treatment strategies. Finally, I hope that this thesis spurs further research in inflammation-induced depression, ultimately leading to new targets for the treatment of "depression due to a general medical condition" and possibly also leading to new targets for the treatment of major depressive disorder in patients that do not respond to regular treatment.

Main observations and conclusions

- LPS-induced reductions in brain stimulation reward are not affected by LPS-induced reductions in motor activity and LPS-induced reductions in appetite and can therefore be interpreted as anhedonia (**Chapter 2** and **Chapter 5**);
- LPS reduces body weight significantly (**Chapter 2** and **Chapter 5**);
- LPS-induced anhedonia is associated with increased levels of monoamine metabolites in the NAc and mPFC (**Chapter 2**, **Chapter 4** and **Chapter 6**);
- TNF-α induces anhedonia, reduces body weight and increases levels of monoamine metabolites in the NAc as well, suggesting that the effects of LPS are probably caused by LPS-induced proinflammatory cytokines (**Chapter 3**);
- Increased monoamine transporter activity is responsible for increased levels of 5-HIAA, DOPAC and HVA in the NAc and mPFC (**Chapter 4**);
- Especially SERT is involved in LPS-induced anhedonia (**Chapter 5** and **Chapter 6**);
- LPS-induced anhedonia is abolished in $SERT^{-/-}$ rats (**Chapter 5**);
- LPS-induced reductions in body weight are less severe in $SERT^{-/-}$ compared to $SERT^{+/+}$ and $SERT^{+/-}$ rats (**Chapter 5**);
- SERT is not involved in LPS-induced cytokine release (**Chapter 6**);
- LPS-induced 5-HIAA formation is abolished in $SERT^{-/-}$ rats (**Chapter 6**);
- Increased SERT activity, deducted from increased 5-HIAA formation, is necessary to induce LPS-induced anhedonia (**Chapter 6**).

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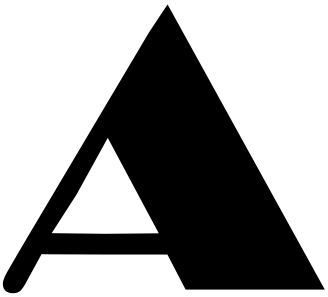

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Samenvatting in het Nederlands

Sinds 30 jaar zijn er steeds meer aanwijzingen dat activatie van het immuunsysteem de kans op een depressie verhoogd. Eerdere studies toonden aan dat cytokinen, dat zijn stoffen die door cellen van het immuunsysteem worden afgegeven, de hersenen bereiken en beïnvloeden. Deze cytokinen verhogen mogelijk de kans op een depressie. De manier waarop dit precies gebeurt is nog niet bekend, maar hier zijn wel ideeën over (**Hoofdstuk 1**). Met meer inzicht in de oorzaak van inflammatie-geïnduceerde depressie kunnen nieuwe antidepressiva ontwikkeld worden die speciaal geschikt zijn voor depressieve mensen met een onderliggende chronisch inflammatoire aandoening.

Een kernsymptoom van depressie is anhedonie. Dit is het onvermogen om plezier te beleven en wordt mogelijk veroorzaakt door een verminderde gevoeligheid van het beloningssysteem in de hersenen. In de meeste experimenten werd lipopolysaccharide (LPS) in de buikholte van ratten en muizen toegediend om het immuunsysteem te activeren. LPS is een essentieel onderdeel van de buitenste membraan van gram negatieve bacteriën en stimuleert cellen van het immuunsysteem tot de afgifte van cytokinen. Met behulp van intracraniale zelf-stimulatie (ICSS) is de gevoeligheid van het beloningssysteem in de hersenen gemeten op verschillende tijdstippen na toediening van LPS om zo inflammatie-geïnduceerde anhedonie in ratten en muizen aan te tonen (**Hoofdstukken 2**, **3**, **4** en **5**).

LPS verminderde zowel bij ratten als muizen de gevoeligheid van het beloningssysteem (**Hoofdstukken 2**, **4** en **5**). De gevoeligheid van het beloningssysteem werd ook verlaagd door tumor necrosis factor α (TNF-α) (**Hoofdstuk 3**). Dit cytokine is na blootstelling aan LPS verhoogd aanwezig in serum (**Hoofdstuk 6**). Dit doet vermoeden dat cytokinen verantwoordelijk zijn voor de LPS-geïnduceerde anhedonie. Daarnaast verlaagde LPS ook de afgelegde afstand in een open veld (**Hoofdstuk 2**). De respons latentietijd, dat is de tijd die het kost voor een dier om in de test te reageren, bleek echter niet te veranderen of juist korter te worden (**Hoofdstukken 2**, **3**, **4** en **5**). Hieruit blijkt dat de LPS- en TNF-α-behandelde dieren even snel of zelfs sneller reageren in de ICSS test, waardoor de gevonden ICSS effecten niet kunnen worden verklaard door het fysieke onvermogen om de test goed uit te voeren. Daarnaast verlaagden LPS en TNF-α het gewicht van ratten en muizen significant (**Hoofdstukken 2**, **3**, **4** en **5**). Dit is te verwachten, aangezien cytokinen de eetlust verminderen. Omdat de ICSS test onafhankelijk is van eetlust en voedselinname, zal dit het LPS- en TNF-α-effect in de ICSS test niet hebben beïnvloed. We zijn er daarom van overtuigd dat de gevonden ICSS resultaten anhedonie weerspiegelen.

Vier uur na blootstelling aan LPS of TNF-α werden de hoogste niveaus van anhedonie gevonden (**Hoofdstukken 2**, **3**, **4** en **5**). Op dit tijdstip zijn cytokinewaarden in serum het hoogst en pieken ook andere symptomen van ziektegedrag, zoals verminderde mobiliteit en verminderde eetlust. Terwijl 24 uur na blootstelling aan LPS de cytokinewaarden weer afnemen en symptomen van ziektegedrag verdwijnen, blijft anhedonie, zoals gemeten in de ICSS procedure, bij ratten tot en met 72 uur na blootstelling aan LPS aanwezig. Het lijkt er dus op dat anhedonie langer duurt dan andere symptomen van ziektegedrag (**Hoofdstuk 2**).

Monoaminen zoals serotonine (5-HT), dopamine (DA) en noradrenaline (NE) zijn verantwoordelijk voor de signaaloverdracht tussen zenuwcellen (neuronen) in de hersenen en spelen een belangrijke rol in de regulatie van emotioneel gedrag. Nadat 5-HT, DA en NE door neuronen zijn afgegeven, binden ze aan receptoren gelegen op nabijgelegen neuronen en aan de afgevende neuronen zelf (autoregulatie). Afhankelijk van het type receptor kunnen ze een stimulerende of remmende werking op dit neuron uitoefenen en op deze manier het gedrag beïnvloeden. De duur van deze signaaloverdracht wordt nauwkeurig gereguleerd door monoaminerge transporters. Serotonine transporters (SERT), dopamine transporters (DAT) en noradrenaline transporters (NET) zorgen ervoor dat respectievelijk 5-HT, DA en NE worden heropgenomen in het neuron. Hiermee wordt signaaloverdracht door middel van 5-HT, DA en NE beëindigd. Huidige antidepressiva zorgen ervoor dat de hoeveelheid monoaminen tussen de neuronen hoog blijft en signaaloverdracht langer duurt. Afwijkingen in de monoaminerge systemen lijken daarom een belangrijke rol te spelen in depressie. Het is interessant om te weten hoe een geactiveerd immuunsysteem monoaminerge systemen (en in het bijzonder de heropname) in het brein beïnvloedt.

Om inzicht te krijgen in de monoaminerge signaaloverdracht tijdens inflammatie-geïnduceerde depressie zijn microdialyse studies uitgevoerd met dezelfde dosering LPS en TNF-α als in de ICSS studies (**Hoofdstukken 2**, **3**, **4** en **6**). Microdialyse is een techniek waarbij in levende dieren monoaminen en metabolieten tussen neuronen in een bepaald hersengebied kunnen worden gemeten, in dit geval de nucleus accumbens en de mediale prefrontale cortex. De werking van beide hersengebieden is verstoord bij depressieve patiënten. De nucleus accumbens speelt een belangrijke rol in beloning en anhedonie, terwijl de mediale prefrontale cortex een belangrijke rol speelt bij het onderdrukken van negatieve emoties.

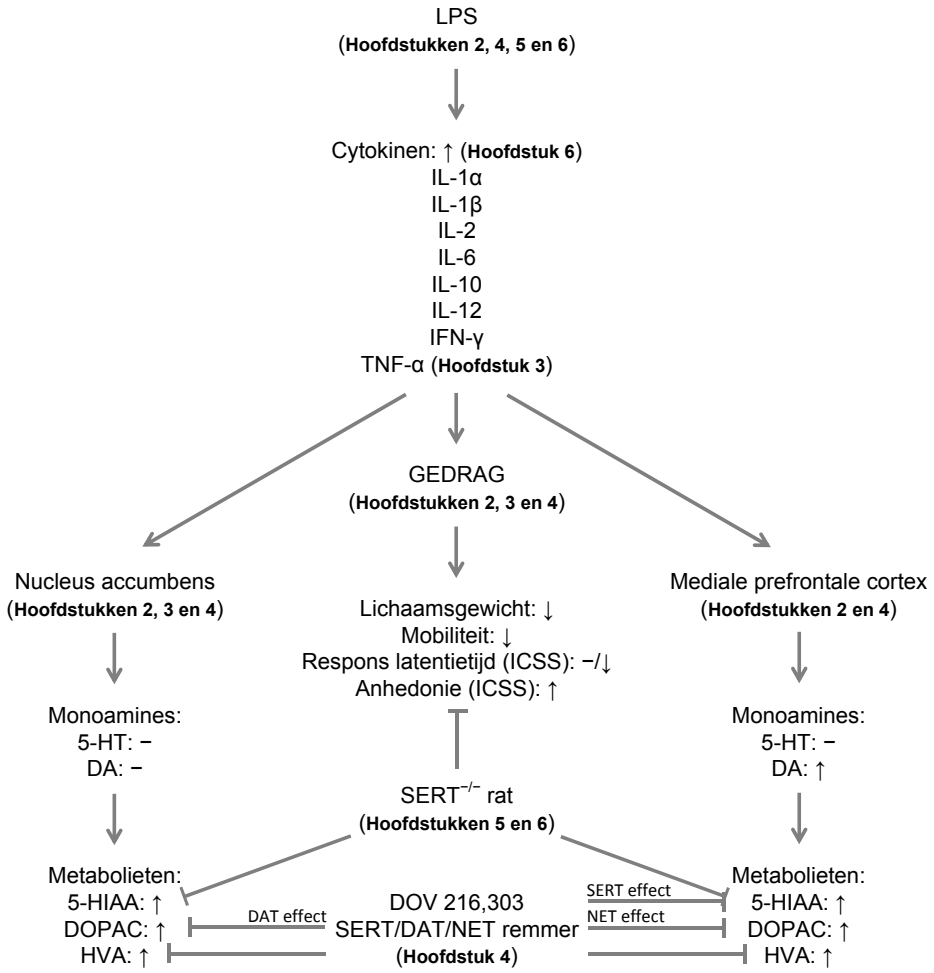
De microdialyse studies toonden aan dat 5-HT en DA niveaus niet daalden na activatie van het immuunsysteem met een dosis van LPS of TNF-α waarvan is aangetoond dat ze anhedonie induceren (**Hoofdstukken 2**, **3**, **4** en **6**). Dopamine steeg zelfs in de mediale prefrontale cortex (**Hoofdstukken 2** en **4**). Dit geeft aan dat anhedonie niet per sé is geassocieerd met tekorten van monoaminen in het brein op de korte termijn.

De metabolieten van 5-HT (5-HIAA) en DA (DOPAC en HVA) stegen significant na blootstelling aan LPS én TNF-α (**Hoofdstukken 2**, **3**, **4** en **6**). Dit suggereert wederom dat de LPS-effecten worden veroorzaakt door LPS-geïnduceerde cytokinen. Een mogelijke bron van verhoogde 5-HIAA en DOPAC (en HVA) niveaus is verhoogde activiteit van monoaminerge transporters. Monoaminerge transporters spelen namelijk niet alleen een belangrijke rol in de beëindiging van het monoaminerge signaal, maar ook in de vorming van monoaminerge metabolieten. Na heropname worden monoaminen voor hergebruik opgeslagen of, en vooral dit laatste is van belang voor dit proefschrift, afgebroken door het enzym monoamine oxidase (MAO). 5-HT wordt afgebroken tot 5-HIAA (zie **Hoofdstuk 1**, ***Figuur 1.2: A***), terwijl DA wordt afgebroken tot DOPAC (zie **Hoofdstuk 1**, ***Figuur 1.2: B***). 5-

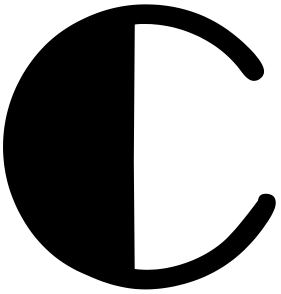
HIAA en DOPAC verlaten vervolgens het neuron. Daarna wordt DOPAC door steuncellen in het centraal zenuwstelsel (gliacellen) verder afgebroken tot HVA (zie **Hoofdstuk 1**, ***Figuur 1.2: D***). 5-HIAA en HVA zijn de hoofdmetabolieten van respectievelijk 5-HT en DA. DA kan echter ook worden afgebroken zonder dat de DAT betrokken is. In dit geval wordt DA in gliacellen afgebroken tot 3-MT en vervolgens tot HVA (zie **Hoofdstuk 1**, ***Figuur 1.2: D***). In de mediale prefrontale cortex zijn DAT niveaus erg laag. DA wordt in dit hersengebied daarom voornamelijk afgebroken in gliacellen of via de NET door noradrenerge neuronen opgenomen om vervolgens tot DOPAC (zie **Hoofdstuk 1**, ***Figuur 1.2: C***) en HVA te worden afgebroken. De LPS-geïnduceerde 5-HIAA, DOPAC en HVA vorming in de nucleus accumbens en mediale prefrontale cortex doet daarom vermoeden dat LPS de SERT, DAT en NET activiteit in de hersenen verhoogd.

LPS-geïnduceerde verhoging van 5-HIAA, DOPAC en HVA werd geremd wanneer de dieren een half uur voor LPS-injectie werden behandeld met de triple reuptake inhibitor DOV 216,303 (**Hoofdstuk 4**). DOV 216,303 remt alle drie transporters: SERT, DAT en NET. Deze microdialyse data suggereren dat LPS de DAT activiteit in de nucleus accumbens en de SERT en NET activiteit in de mediale prefrontale cortex verhoogd. Aangezien de gebruikte dosering van LPS anhedonie induceerde (**Hoofdstuk 4**), doet dit vermoeden dat monoaminerge transporter activiteit een rol speelt in inflammatie-geïnduceerde anhedonie. Daarnaast werd aangetoond dat ratten zonder SERT ($SERT^{-/-}$ ratten) geen LPS-geïnduceerde anhedonie laten zien in de ICSS procedure (**Hoofdstuk 5**). Dit geeft aan dat voornamelijk SERT belangrijk is in inflammatie-geïnduceerde anhedonie. Bovendien verloren deze dieren veel minder gewicht (**Hoofdstuk 5**) en lieten ze geen LPS-geïnduceerde 5-HIAA vorming zien (**Hoofdstuk 6**). Dit laatste geeft aan dat LPS-geïnduceerde 5-HIAA niveaus zeer waarschijnlijk worden veroorzaakt door verhoogde SERT activiteit. Interessant is dat $SERT^{-/-}$ ratten geen afwijkende cytokine respons lieten zien (**Hoofdstuk 6**). Dit geeft aan dat de immuunrespons van $SERT^{-/-}$ dieren niet afwijkt van ratten waarbij SERT wel actief is.

In **Hoofdstuk 7** worden de resultaten eerst samengevat (zie ook ***Figuur S.1***) en naast de literatuur gelegd. Ook worden tekortkomingen en ideeën voor toekomstig onderzoek besproken. Het belang van screening voor depressie bij patiënten met chronisch inflammatoire aandoeningen wordt aangegeven. De bevindingen die worden gepresenteerd in dit proefschrift bieden mogelijkheden voor de behandeling van depressies die zijn geassocieerd met hoge niveaus van proinflammatoire cytokinen in serum. Omdat monoaminerge transporter activiteit niet de enige route is via welke deze cytokinen een depressie kunnen induceren, wordt gewezen op het belang van toekomstig onderzoek naar alle mechanismen die betrokken zijn bij inflammatie-geïnduceerde depressie. Hopelijk leidt dit uiteindelijk tot nieuwe aangrijpingspunten voor de behandeling van depressie met een onderliggende chronisch inflammatoire aandoening.

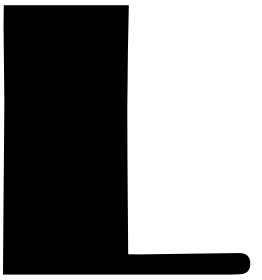


Figuur S.1: Schematisch overzicht van de bevindingen gepresenteerd in dit proefschrift. Lipopolysaccharide (LPS) toegediend in de buikholte leidt tot een toename van cytokinen in serum en brein. Deze cytokinen, waaronder tumor necrosis factor α (TNF-α), bereiken het brein en veranderen het gedrag: lichaamsgewicht neemt af door verminderde eetlust, mobiliteit wordt minder, respons latentietijden blijven onveranderd of gaan omlaag en de dieren vertonen anhedonisch gedrag in de intracraniale zelfs-stimulatie (ICSS) test. De microdialyse studies tonen aan dat serotonine (5-HT) en dopamine (DA) gehaltes niet veranderen na toediening van LPS of TNF-α. Alleen in de mediale prefrontale cortex neemt DA toe. De metabolieten van 5-HT en DA stijgen in beide hersengebieden. Voorbehandeling met DOV 216,303, een remmer van de serotonine transporter (SERT), dopamine transporter (DAT) en noradrenaline transporter (NET), verhindert de LPS-geïnduceerde metabolietvorming in beide hersengebieden. Dit suggereert dat monoaminerge transporter activiteit toeneemt onder invloed van LPS. Voornamelijk lijkt toegenomen SERT activiteit betrokken bij LPS-geïnduceerde anhedonie, aangezien $SERT^{-/-}$ ratten niet gevoelig zijn voor LPS-geïnduceerde anhedonie. Bovendien laten deze dieren ook geen significante toename in 5-HIAA metabolietvorming zien wat aangeeft dat toegenomen 5-HIAA levels een indicatie geven voor toegenomen SERT-activiteit.



Curriculum vitae

Floor van Heesch was born on 15 April 1986 in Nijmegen, The Netherlands. In 2004 she received her Atheneum diploma from the Nijmeegse Scholengemeenschap Groenewoud (NSG) in Nijmegen. That same year she started the bachelor's programme in Biomedical Siences at the Radboud University Nijmegen. During her bachelor's study, she conducted a research project at the department of Chemical Endocrinology of the Radboud University Nijmegen Medical Centre under supervision of Dr. Paul N. Span. The project was entitled "Tribbles 3 and radiotherapy sensitivity in relation with inhibition of the HER2/TK/PI3Kinase pathway". After graduating for her bachelor's degree in 2007 Floor started the master Human Pathobiology. During her minor internship she investigated the role of alternative signaling of TGF-beta via ALK1 instead of ALK5 in chondrocyte differentiation and its potential implication for osteoarthritis development. This internship was performed under supervision of Dr. Esmeralda N. Blaney Davidson and Dr. Peter M. van der Kraan at the laboratory of Rheumatology Research & Advanced Therapeutics of the Nijmegen Centre of Molecular Life Sciences (NCMLS). During her master internship, Floor was supervised by Dr. Jocelien D.A. Olivier and Dr. Judith R. Homberg at the department of Cognitive Neuroscience of the UMC St Radboud Nijmegen on the project entitled "Fluoxetine treatment during pregnancy affects behavior of the offspring – The paradoxical effects of prenatal exposure to fluoxetine". In 2009 Floor graduated bene meritum from Radboud University Nijmegen with an MSc in Biomedical Sciences. Since November 2009 Floor was employed as a PhD-student under supervision of Dr. S. Mechiel Korte, Dr. Aletta D. Kraneveld and Prof. Dr. Berend Olivier at the department of Pharmacology at Utrecht University. Most of the results obtained in this project are described and discussed in this thesis.



List of publications

Journal articles (first author)

van Heesch, F., Prins, J., Konsman, J.P., Westphal, K.G., Olivier, B., Kraneveld, A.D., Korte, S.M., 2013. Lipopolysaccharide-induced anhedonia is abolished in male serotonin transporter knockout rats: An intracranial self-stimulation study. Brain Behav. Immun. 29, 98-103.

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Journal articles (coauthor)

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Abstracts

van Heesch, F., Westphal, K.G.C., Prins, J., Olivier, B., Kraneveld, A.D., Korte, S.M., 2011. Peripheral LPS challenge affects monoamine neurotransmitter levels in the nucleus accumbens. Pharma-Nutrition conference, Amsterdam, The Netherlands.

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van Heesch, F., Westphal, K.G.C., Korte-Bouws, G.A.H., Prins, J., Olivier, B., Kraneveld, A.D., Korte, S.M., 2011. Peripheral LPS challenge affects monoaminergic neurotransmission in the nucleus accumbens. Anxiety and Depression: 21st Neuropharmacology Conference, Washington, U.S.A.

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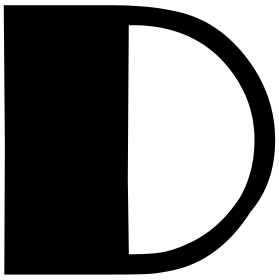

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